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IMMUNOLOGICAL AND BIOSYNTHETIC STUDIES

ON TWO MITOCHONDRIAL DEHYDROGENASES

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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February, 1987.

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ACKNOWLEDGEMENTS

I would like to thank Professor R.M.S. Smellie for making available the facilities necessary for the successful completion of this work. I also acknowledge the financial support of the Science and Engineering Research Council.

I would like to thank my Supervisor, Dr. J. Gordon Lindsay, for his friendly and patient guidance, and for encouraging me to cross the Atlantic; also the members of labs C-35 and C-36 for their friendship and support.

The following people also deserve special mention; Dr. Steve Yeaman (University of Newcastle Upon Tyne), for providing samples of purified bovine kidney branched chain 2-oxoacid dehydrogenase and E1 subcomplex; Professor Tsoo King (State University of New York at Albany) for providing the succinate dehydrogenase preparation used in this study; Dr. Bob Eason and Messrs Tom Mathieson and Kenneth Ensor for their help in using the word processing facilities; also the staff of the Departmental Animal House, Wellcome Tissue Culture Unit and Medical Illustration Unit for their assistance.

Finally, I would like to thank my parents for the support and encouragement they have given me during my years at University, both in Glasgow and in Edinburgh.

I typed this thesis myself on a Videcom Apollo Desk Top Computer using WORDSTAR and printed it using a Toshiba TH-2100H printer.

ABBREVIATIONS

The abbreviations used throughout this Thesis are those recommended by the Biochemical Journal in its Instructions to Authors (Biochem. J. (1986) 233, 3-21) with the following additions:-

CoASH, coenzyme A (reduced form)

BCOAD, branched chain 2-oxoacid dehydrogenase

BRL, Buffalo rat liver

BSA, bovine serum albumin

CCCP, carbonyl cyanide, m-chlorophenyl hydrazone

DHFR, dihydrofolate reductase

DOC, deoxycholate, sodium salt

DNP, 2,4-dinitrophenol

DTT, dithiothreitol

ER, endoplasmic reticulum

FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone

Iodogen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril

Leupeptin, acetyl-L-leucyl-L-leucyl-L-argininal

LMM, low methionine medium

MMM, minus methionine medium

MSUD, maple syrup urine disease

NBL-1 bovine kidney

NEM, N-ethylmaleimide

NGM, normal growth medium

OGDC, 2-oxoglutarate dehydrogenase complex

PBS, phosphate buffered saline

PEG, polyethylene glycol

PDH, pyruvate dehydrogenase complex

PK-15, pig kidney

PMSF, phenylmethylsulphonylfluoride

PPO, 2,5-diphenyloxazole

SDH, succinate dehydrogenase

SRP, signal recognition particle

TEMED, N,N,N',N'-tetramethylethylenediamine

TLCK, N-α-ρ-tosyl-L-lysine-chloromethyl ketone

TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone

TPP, thiamine pyrophosphate

Tween 20, polyethylenesorbitan monolaureate

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SUMMARY

Monospecific, polyclonal antisera were raised to purified bovine heart succinate dehydrogenase (SDH), and to the individual large (M_r = 70000) and small (M_r = 27000) subunits of this enzyme. The specificities of the antisera were determined by immune blotting. These antisera exhibited cross-reactivity with the corresponding polypeptides in Buffalo rat liver (BRL), pig kidney (PK-15) and bovine kidney (NBL-1) cell lines, and were employed to investigate some of the events involved in the biogenesis of succinate dehydrogenase in the BRL and PK-15 cell lines.

Newly-synthesised forms of the large and small subunits of SDH were detected in cultured PK-15 and BRL cells labelled with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation. In both cell lines, the precursor forms of the large and small subunits exhibit M_r values approx. 1-2000 and 4-5000 greater than the corresponding mature forms. When the uncoupler is removed in pulse-chase experiments, complete conversion of the precursor to the mature forms occurs within 45 min.

Studies on the kinetics of processing of the large subunit precursor revealed that reversal of precursor accumulation is rapid, with processing occurring with a half-time of 5-7.5 min. The accumulated precursor exhibits long term stability when PK-15 cells are maintained in the presence of DNP.

The arrangement of the large and small subunits of succinate dehydrogenase on the mitochondrial inner membrane was investigated by immune blot analysis of protease-treated bovine heart mitochondria

(right side-out; outer membrane removed) or submitochondrial particles (inside-out). Both subunits were found to be absent from the cytoplasmic surface of the inner membrane. The bulk of the small subunit appears to protrude into the matrix compartment, since this subunit is degraded during protease treatment of submitochondrial particles, without the appearance of a membrane-associated fragment. The data obtained in this study suggests that the large subunit may interact with the matrix side of the inner membrane via two distinct regions; these are detected as membrane-associated fragments of M_r 32000 and 27000 after treatment of submitochondrial particles with papain or protease K.

An analogous biosynthetic study was performed on the mammalian branched chain 2-oxoacid dehydrogenase (BCOAD) multienzyme complex, a high-M_r assembly composed of multiple copies of a branched chain 2-oxoacid dehydrogenase (E1), dihydrolipoamide acyltransferase (E2) and lipoamide dehydrogenase (E3). This was achieved using antisera raised to (a) the purified complex from bovine kidney (anti-BCOAD serum); (b) the purified E1 subcomplex and (c) the E2 subunit isolated by preparative SDS/polyacrylamide gel electrophoresis. Both anti-BCOAD and anti-E1 sera exhibited a relatively low titre of antibodies to the E1β polypeptide. An unexpected finding of this study was that the lipoamide dehydrogenase (E3) component was present in immunoprecipitates of the PK-15 BCOAD complex which were obtained using an antiserum which lacked antibodies against E3. This finding suggests that the absence of E3 from most preparations of purified BCOAD complex does not reflect the physiological situation but is a result of the isolation procedure.

The precursor form of the BCOAD complex E1 subunit was detected in the pig kidney and bovine kidney cell lines. This species exhibited a M_r value which was approx. 3000 greater than the mature-sized polypeptide. In contrast, the newly-synthesised E1β subunit evaded detection, largely due to the low immunogenicity of this polypeptide. The precursor form of the BCOAD complex E2 subunit was detected as a 56000-M_r species and was therefore similar in size to the previously identified pre-E3 polypeptide. However, the separate identity of the M_r 56000 species and the E3 precursor was confirmed in immunocompetition experiments using unlabelled purified pig heart E3. The presequence of the BCOAD complex E2 precursor is markedly smaller than those of the E2 precursors of the analogous 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, which both have M_r values of approx. 8000.

CHAPTER ONE

INTRODUCTION

1.1 PROTEIN TARGETTING

1.1.1 TARGETTING OF SECRETORY PROTEINS

In recent years, attention has increasingly been focused on the phenomenon of "protein targetting", i.e. the distribution of polypeptides from their cytoplasmic site of synthesis to their correct subcellular locations.

Initial insights into this area of Cell Biology were obtained from studies on proteins which are targetted to the extracellular space (secretory proteins). Using mainly pancreatic and liver cells (two cell types highly specialised for protein export), it was found that secretory proteins were produced on ribosomes bound to the endoplasmic reticulum (ER), and that newly-synthesised proteins did not appear in the cytosol but were transferred directly into the lumen of the ER *in vivo* and of microsomal vesicles *in vitro* (Redman *et al*., 1966; Chefurka and Hayashi, 1966). The authenticity of the latter observation was substantiated by Redman and Sabatini (1966), who demonstrated that even incomplete polypeptide chains, obtained using puromycin, were not located on the cytoplasmic side of the ER membrane. It was then shown that microsomes protect nascent chains of secretory but not of cytoplasmic proteins from proteolytic degradation (Sabatini and Blobel, 1970). These observations led to the concept that polypeptide chains synthesised by ribosomes bound to the ER are discharged vectorially through the membrane.

Several reasons have been put forward to explain the presence of a particular class of mRNAs (mainly those for secretory proteins) in membrane-bound polysomes. The segregation of this class of mRNAs was proposed to occur via: 1) binding of incomplete initiation complexes

composed of mRNA and 40S ribosomal subunits to membrane-bound 60S subunits (Baglioni *et al.*, 1971); 2) formation of a specific link between the mRNA and the membrane (Mechler and Vassalli, 1975); 3) attachment of bound polysomes via the N-terminal region of the growing polypeptide chain (Blobel and Sabatini, 1971).

Evidence for the third possibility was first presented by Milstein *et al.* (1972), who studied the *in vitro* translation of immunoglobulin light chain mRNA. These authors showed that a larger precursor containing extra amino acids at the N-terminus was synthesised in a reticulocyte lysate, but not in cell-free translation systems containing microsomal membranes. Similar higher M_r precursors, only detectable *in vitro* in the absence of microsomes, were subsequently described for parathyroid hormone (Kemper *et al.*, 1974), placental lactogen (Boime *et al.*, 1975) and melittin (Suchanek *et al.*, 1975).

These observations, together with the work of Blobel and Dobberstein (1975a,b) on the translocation of nascent immunoglobin light chains across microsomal membranes, led to the formation of the signal hypothesis. In its earliest form, this hypothesis made the following predictions; a) secretory polypeptides contain an N-terminal signal peptide which binds to the ER and initiates vectorial discharge of the growing polypeptide chain; b) this transport (and therefore binding) must start early, before the chain reaches a certain length, and be strictly coupled to protein synthesis; c) signal peptides are transient species which are removed by proteolytic cleavage before chain completion.

Since the advent of this hypothesis, progress has been made in obtaining a more detailed understanding of the molecular interactions

required for vectorial transport (reviewed by Walter _et al_., 1984). An updated version of the model is depicted in Fig.1.1.

It is now recognised that at least two components are required for the translocation event. The first of these is the signal recognition particle (SRP), an 11S cytoplasmic ribonucleoprotein consisting of six nonidentical polypeptides with M_r values of between 72000 and 9000 (Walter and Blobel, 1980) and one molecule of 7S RNA (Walter and Blobel, 1982). The SRP functions by recognising and binding to a nascent signal sequence once it has emerged from the 60S ribosomal subunit, and thus temporarily arrests translation (Walter and Blobel, 1981).

The second isolated component of the protein translocation machinery is the SRP receptor (Gilmore _et al_., 1982) or docking protein (Meyer _et al_., 1982); a 72000-M_r integral ER membrane protein which apparently mediates the interaction of SRP-arrested ribosomes with the endoplasmic reticulum. By virtue of this SRP-docking protein interaction, translation of the nascent polypeptide resumes and translocation into the lumen of the ER commences (Gilmore _et al_., 1982).

The steps following this initial targetting event are relatively poorly understood. Once targetting to the ER surface has occurred, the ribosome-SRP-docking protein interaction is postulated to be replaced by a direct interaction of the ribosome with other integral membrane proteins (Walter _et al_., 1984). Two ER-specific proteins, termed ribophorins I and II (Kreibich _et al_., 1978a,b) may be involved in this type of interaction.

Once a nascent protein has penetrated the ER membrane, its signal peptide is proteolytically removed by a specific integral membrane

Fig.1.1 Current Picture Of The Events Occurring During The Translocation Of Polypeptides Across The Endoplasmic Reticulum (after Walter *et al.*, 1984).

a; free (soluble) SRP

b; ribosome-bound SRP

c; translational arrest

d; SRP-docking protein interaction

e; membrane-bound SRP

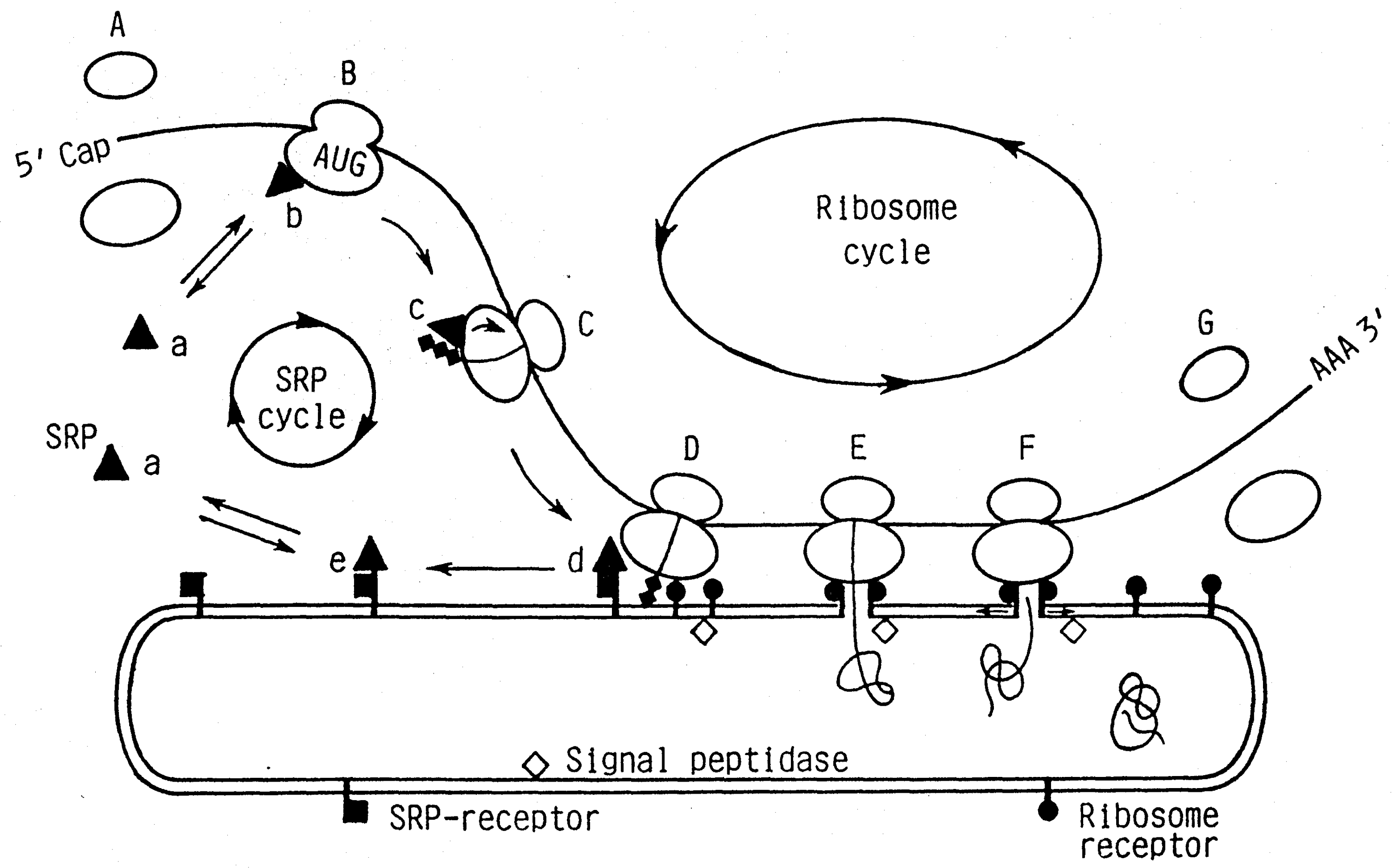
A-C; translation of mRNA encoding a signal sequence addressed to the ER protein translocating system

D; interaction of SRP-arrested ribosomes with microsomal membrane

E; translocation of nascent polypeptide through membrane pore

F; release of completed polypeptide into lumen of ER

G; release of ribosome from the membrane



A
B
5' Cap
AUG
b
a
SRP cycle
SRP
a
c
C
Ribosome cycle
G
AAA 3'
D
E
F
e
d
Signal peptidase
SRP-receptor
Ribosome receptor

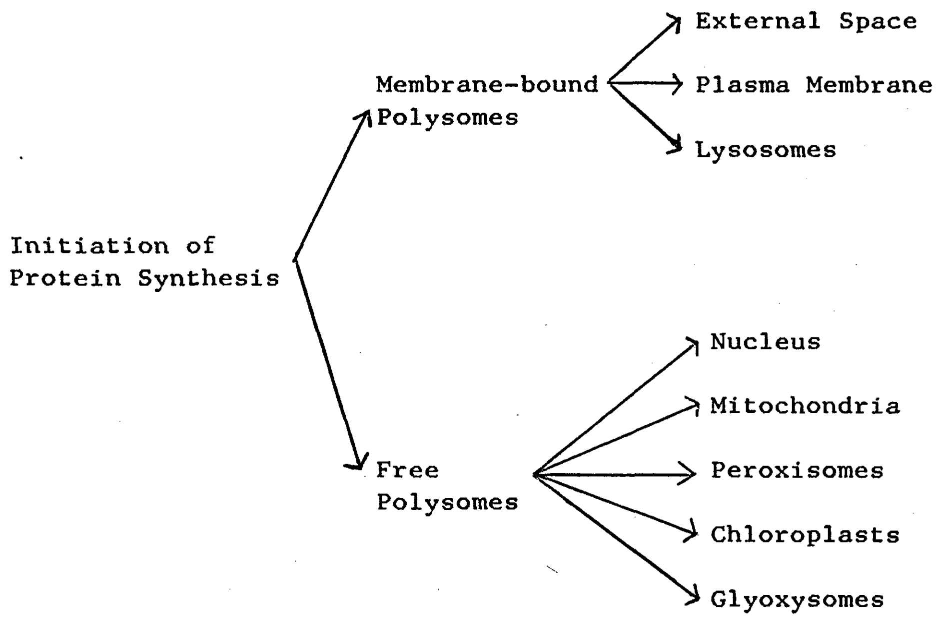
endoprotease, termed the signal peptidase, which is located at the lumenal side of the membrane (Jackson and Blobel, 1977). Very recently, Evans et al. (1986) have reported the purification of signal peptidase from canine pancreatic microsomes. The purified enzyme described by these workers consists of a complex of six different polypeptides. However, the authors suggest that only one subunit is responsible for signal peptide removal, and that the structural association of the other subunits in stoicheiometric amounts may reflect their requirement in chain translocation; for instance they may form part or all of a hypothetical pore through which the nascent chain is proposed to cross the membrane (Blobel and Dobberstein, 1975a). In the case of integral membrane proteins the translocation process is aborted prior to completion of the polypetide chain, resulting in its asymmetrical integration into the membrane. The information causing the arrest of translocation (a putative stop-transfer sequence) is most likely contained in the nascent chain and interpreted by the translocation machinery.

For many proteins which are transferred across the lipid bilayer of the endoplasmic reticulum, translocation is the first in a series of events which effect their transfer to locations such as the lysosomes, Golgi apparatus, plasma membrane, or the extracellular space.

1.1.2 DISTRIBUTION OF NON-SECRETORY TARGETTED PROTEINS

The cotranslational, ER-mediated type of protein translocation described above for secretory proteins is also involved in the targetting of lysosomal proteins (Erickson et al., 1981) and of certain classes of integral membrane proteins (Lingappa et al., 1978).

Fig.1.2 General Scheme For Distribution Of Proteins In Eukaryotes



Primary Distribution

Secondary Distribution

However, when the question of how cytoplasmically-synthesised polypeptides are translocated across the membranes of organelles such as chloroplasts, mitochondria, peroxisomes and glyoxysomes was addressed, it became clear that cotranslational transport was not the sole mechanism for translocation of proteins across membranes.

Initial approaches involved the use of cell-free protein synthesis systems for characterising the primary translation products of mRNAs that direct cytoplasmic synthesis of polypeptides destined for specific organelles. Using mRNA isolated from the unicellular green alga, *Chlamydomonas reinhardti*, Dobberstein *et al*. (1976) demonstrated that the small subunit of the chloroplast enzyme ribulose 1,5 bisphosphate carboxylase was synthesised as a higher M_r precursor, which could be converted to its mature form after completion of the polypeptide chain. In addition, it was found that the mRNA encoding the small subunit precursor was localised in preparations of free polysomes. These findings suggested that the small subunit precursor is transferred through the two chloroplast envelope membranes by a posttranslational mechanism. Supporting evidence for posttranslational transport of chloroplast proteins was subsequently provided (Chua and Schmidt, 1978; Highfield and Ellis, 1978). Later studies on the biosynthesis of mitochondrial, peroxisomal and glyoxysomal proteins (Maccecchini *et al*., 1979; Robbi and Lazarow, 1978; Zimmermann and Neupert, 1980) revealed that polypeptides were also transported into these organelles by a similar posttranslational mechanism. Our current understanding of the mode of distribution of cytoplasmically-synthesised polypeptides is summarised in Fig.1.2.

1.2 BIOGENESIS OF MITOCHONDRIA

1.2.1 PERSPECTIVES

Mitochondria, like chloroplasts, are distinct among cellular organelles in that they possess an extranuclear genetic system. However, the information content of mitochondrial DNA is insufficient to account for the estimated several hundred different polypeptides which are found in a functional mitochondrion (cf. section 1.2.2). Indeed, synthesis of the full repertoire of mitochondrial polypeptides depends on the close cooperation of the mitochondrial and nucleocytoplasmic genetic systems, with the latter producing approximately 95% of all mitochondrial polypeptides. There is evidence to suggest that this cooperation is mediated by the protein products of both genetic systems (Felipo and Grisolia, 1984), although it is likely to occur at many different levels.

All the available data, and theoretical considerations, suggest that cellular membranes do not form _de novo_, but through the growth and division of pre-existing structures (Sitte, 1980; Yaffe and Schatz, 1984). Therefore, the biogenesis of mitochondria involves incorporation of many different cytoplasmically-synthesised polypeptides (as well as other components such as lipids) into pre-existing organelles. Moreover, mitochondria contain two membranes which give rise to four submitochondrial locations; the outer membrane, inner membrane, the intermembrane space, and the matrix, which is enclosed by the inner membrane. Since each of these submitochondrial locations contains a defined set of polypeptides (Ernster and Kuylenstierna, 1970), the incorporation process must be highly specific, and be mediated by an intramitochondrial sorting mechanism.

As illustrated in Fig.1.3, the biogenetic pathway pertaining to a cytoplasmically-synthesised mitochondrial polypeptide can be divided into five steps;

1) synthesis of the polypeptide (usually as a higher M_r precursor).

2) delivery and binding of the precursor to the mitochondrial surface.

3) translocation of the precursor across or into one or both mitochondrial membranes, depending on the final submitochondrial location of the polypeptide.

4) cleavage and/or other covalent modifications (processing) of the polypeptide chain to generate the mature protein.

5) assembly of mature polypeptides into functional holoenzymes, which in some cases involves their association with mitochondrial gene products.

1.2.2 THE MITOCHONDRIAL GENOME

All eukaryotes examined to date contain within their mitochondria multiple copies of an organelle-specific, double-stranded DNA molecule, which encodes several mitochondrial RNA and polypeptide components. Mitochondrial DNA ranges in size from approximately 15 kilobase pairs in animals to 600-750 kilobase pairs in some higher plants. Much of this diversity is due to differences in the efficiency with which coding information is packed into the genome (Borst *et al.*, 1984). The complete nucleotide sequences of the human, bovine, mouse and *Xenopus laevis* mitochondrial genomes have recently been determined (Anderson *et al.*, 1981; Anderson *et al.*, 1982; Bibb *et al.*, 1981; Roe *et al.*, 1985). These genomes are very similar in size and show the

Fig.1.3 Steps In The Biogenetic Pathway Of An Imported Mitochondrial Polypeptide

1; Precursor synthesis.

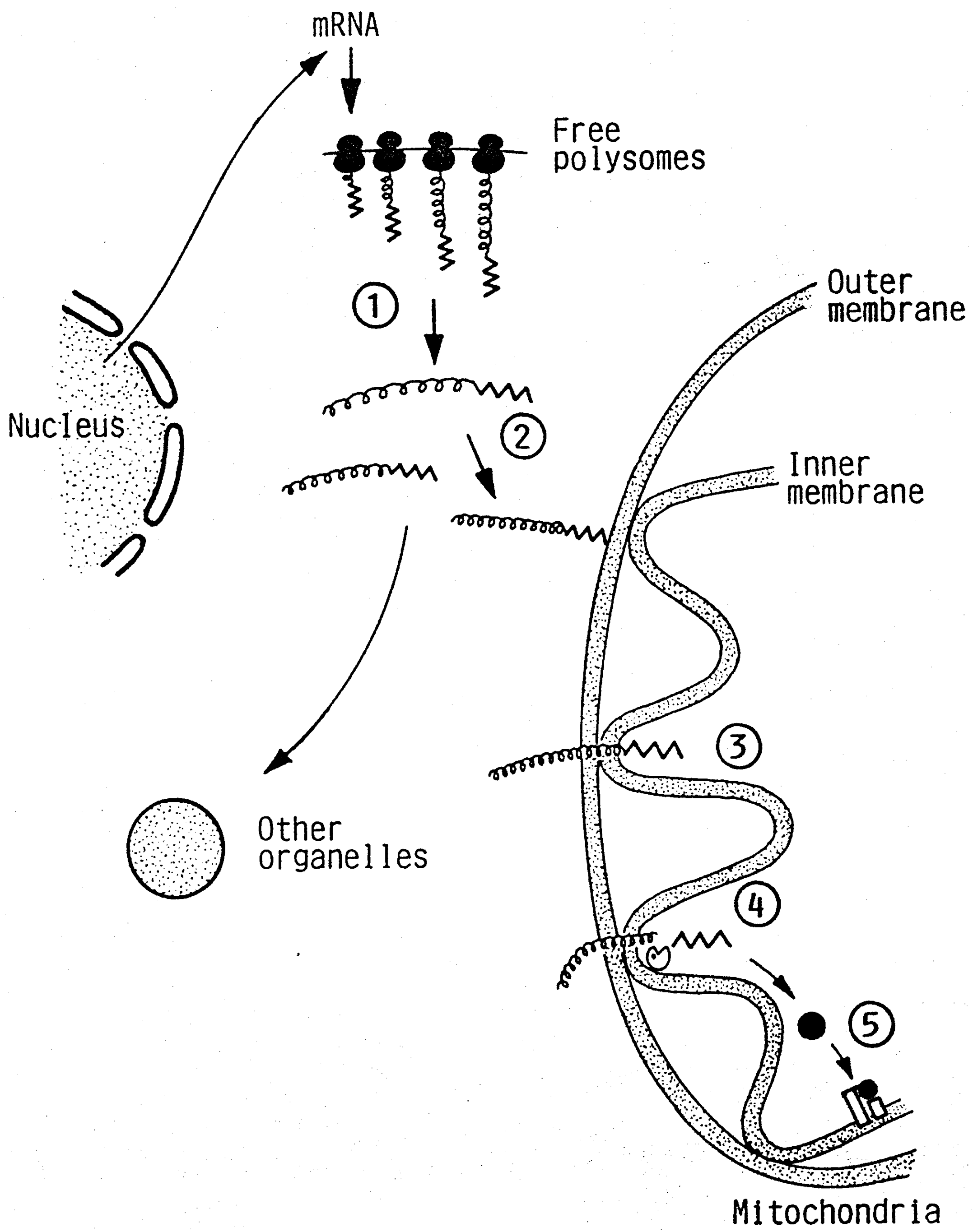
2; Delivery and binding of precursor to the mitochondrial surface.

3; Translocation.

4; Processing.

5; Refolding and assembly into functional units.

The N-terminal extension is represented by a zig-zag line and the mature sequence by a spiral.


mRNA
Free polysomes
Nucleus
1
2
Outer membrane
Inner membrane
3
Other organelles
4
5
Mitochondria

same organisation and complement of genes that code for highly homologous versions of 22 tRNAs, two ribosomal RNAs, and 13 proteins. The two rRNA genes and 14 of the tRNA genes are located on the heavy (H) strand, as are 12 of the 13 protein-coding genes. Eight tRNA genes and one protein-coding gene are located on the light (L) strand.

Five of the protein-coding genes have been identified unambiguously as those for subunits I, II and III of cytochrome c oxidase, ATPase subunit 6, and cytochrome b. Moreover, a recent report by Chomyn et al. (1985) provides evidence that six of the remaining unidentified reading frames in human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. These findings imply that at least four multi-subunit enzymes of the mitochondrial inner membrane are formed through the physical association of mitochondrial and nuclear gene products.

The organisation of the mitochondrial genome in higher eukaryotes is extremely economical. Genes for the 22 tRNAs are interspersed between the ribosomal RNA and protein coding regions, possibly serving as punctuation points for processing of the primary transcripts. With the exception of the displacement loop region, there are very few noncoding nucleotides and no apparent introns. These features are indicative of an extremely specialised genome with little opportunity for further simplification.

In many organisms the mitochondrial genetic code differs from the "universal" code utilised by nucleocytoplasmic and prokaryotic genetic systems, with different organisms using different variations. For instance, the code in mammalian mitochondria uses AGA and AGG as termination rather than Arg codons. The "universal" UAA termination codon is also used by mammalian mitochondria, although, in many cases,

this is generated by polyadenylation of pre-mRNAs which end in U or UA following their cleavage from the primary transcript (Anderson *et al*., 1981). Another common variation, found in the mitochondrial genetic code of higher eukaryotes, *Saccharomyces cerevisiae* (referred to hereafter as yeast), *Neurospora crassa* and *Aspergillus nidulans* is the use of the "universal" UGA stop codon as an additional Trp codon. The aforementioned economy of organisation of the mammalian mitochondrial genome is augmented by the existence of mitochondrial tRNA molecules which can recognise as many as four synonymous codons, rather than the two codons recognised by conventional tRNAs. Thus, the faithful reading of all codons in mitochondrial mRNAs is accomplished by only 22 tRNAs. This finding has been substantiated by primary sequence analysis of several mitochondrially-synthesised polypeptides (Anderson *et al*., 1981).

1.2.3 PRECURSOR FORMS OF MITOCHONDRIAL POLYPEPTIDES

1.2.3.1 SITE OF SYNTHESIS OF PRECURSOR POLYPEPTIDES

Elucidation of which subcellular sites are involved in the synthesis of nuclear coded mitochondrial polypeptides has been crucial to an understanding of how mitochondria import these species *in vivo*. Early studies which addressed this question looked at the biosynthesis of mitochondrial proteins such as cytochrome c and glutamate dehydrogenase (GDH) *in vivo* (Gonzalez-Cadavid, 1974; Kawajiri *et al*., 1977). In these experiments, animals were injected with radiolabelled amino acids and the distribution of labelled proteins into various subcellular fractions was monitored. In general, the highest specific activities of the precursors at the earliest time points were found in

the microsomal fractions.

The site of synthesis of glutamate dehydrogenase was also studied by isolating free and membrane-bound polysomes from the livers of rats injected with [^{3}H]leucine. Nascent chains released from the ribosomes were screened for nascent GDH with pepsin-digested antibodies to GDH. A preferential reaction of anti-GDH antibodies with nascent chains from membrane-associated polysomes was observed. This data suggested that synthesis of mitochondrial precursors occurred on polysomes associated with microsomal membranes.

However, there are now serious reservations which question whether these findings have physiological significance, or reflect artefacts of subcellular fractionation techniques. The first concerns the extent of selective redistribution of newly-synthesised mitochondrial proteins during tissue homogenisation. For instance, Godinot and Lardy (1973) showed that radiolabelled GDH added to liver homogenates becomes associated with microsomal membranes in an apparently non-specific manner. Moreover, Robbi _et al_. (1978) demonstrated that newly-synthesised cytochrome _c_ was selectively released from mitochondria upon liver homogenisation and became bound to liver microsomes.

The second concerns the immune assays on nascent polypeptides associated with polysomes. Nascent chains tend to form aggregates and thus could cause non-specific precipitation. Indeed, the work of Kawajiri _et al_. (1977) did not provide rigorous proof that the immunoprecipitated nascent chains were related to GDH. Furthermore, Hay _et al_. (1984) were unable to demonstrate either qualitative or quantitative changes in the rate of synthesis of several mitochondrial proteins or accumulation of their precursor forms by pulse-labelling temperature-sensitive mutants in the yeast secretory pathway at a

nonpermissive temperature. This finding suggests that any role of the secretory pathway in mitochondrial biogenesis is at most a minor one.

The most recent investigations of the subcellular site of synthesis of mitochondrial proteins have employed an alternative strategy to the pulse-labelling approach described above. Polysomes (either free or extracted from microsomes) or mRNA extracted from these polysomes were translated in cell-free systems, then the translation products were screened immunologically for specific mitochondrial proteins. Using this approach, the translation of cytochrome c, porin, and subunit 9 of the ATPase of *N. crassa* (Zimmermann *et al.*, 1979; Freitag *et al.*, 1982; Schmidt *et al.*, 1983b), the outer membrane protein OMM35 of rat liver and several matrix enzymes of higher eukaryotes (Raymond and Shore, 1979; Morita *et al.*, 1981; Shore *et al.*, 1981; Mihara *et al.*, 1982b; Sonderegger *et al.*, 1982) and of twelve mitochondrial precursors in yeast (Suissa and Schatz, 1982) occurs predominantly on free ribosomes. This suggests that, for these precursors at least, import into mitochondria occurs posttranslationally.

Additional evidence for a posttranslational import mechanism *in vivo* comes from the fractionation of pulse-labelled yeast spheroplasts, fibroblasts and hepatocytes. After a short pulse with [^{35}S]methionine, labelled precursor forms of mitochondrial polypeptides are found in the cytosol, whereas mature forms are found inside mitochondria, protected from externally-added protease (Reid and Schatz, 1982; Jaussi *et al.*, 1981; Mori *et al.*, 1981). The mature intramitochondrial form appears only after a lag period, indicating the existence of a cytoplasmic pool of precursor molecules.

In apparent contradiction to a posttranslational mechanism for

protein import, cytoplasmic ribosomes have been found attached to the mitochondrial outer membrane in cycloheximide-treated yeast cells (Kellems and Butow, 1972; Kellems *et al*., 1974; Ades and Butow, 1980). When the mRNA extracted from these mitochondrially-associated polysomes was translated *in vitro* and compared with mRNA extracted from "free" cytoplasmic polysomes, it was found to be specifically enriched for mRNAs of mitochondrial proteins (Suissa and Schatz, 1982). In none of the twelve cases examined, however, was the total amount of mRNA for a particular mitochondrial polypeptide found to be predominantly or exclusively localised to the polysomes bound to mitochondria.

These observations suggest that if elongation of a mitochondrial polypeptide is inhibited (as with cycloheximide), some nascent polypeptide chains together with the attached polysome may bind to mitochondria. Whether the nascent protein chains are actually integrated into the mitochondrion prior to polypeptide completion remains to be settled. However, there is no firm evidence pointing to an obligatory role for cotranslational translocation (see section 1.1.1) in the import of mitochondrial polypeptides.

1.2.3.2 MOLECULAR CHARACTERISTICS OF PRECURSOR MOLECULES

The identification of mitochondrial polypeptide precursors and of the events involved in their uptake into mitochondria has relied heavily on two principal approaches. One of these is an *in vitro* approach, in which isolated mRNA is translated in a cell-free system in the presence of a radioactive amino acid. The precursor form of a mitochondrial polypeptide can be immunoprecipitated from the

translation mixture using antibodies against its mature counterpart, provided that the precursor is also recognised by these antibodies. In most cases, the precursor form is observed as a band which migrates more slowly upon SDS/polyacrylamide gel electrophoresis than does the mature polypeptide (see below).

Posttranslational import can be accomplished and detected *in vitro* by adding isolated intact mitochondria to the cell-free system after translation has occurred, and immunoprecipitating imported (mature) polypeptides from reisolated mitochondria. The intramitochondrial location of radiolabelled polypeptides can be verified by demonstrating that they are resistant to externally-added protease.

An alternative *in vivo* approach also employs a highly specific antiserum against a mature polypeptide to recognise its precursor form. Whole cells are subjected to "pulse" or "pulse-chase" labelling with a radioactive amino acid, then immunoprecipitation is performed on cytosolic and mitochondrial fractions, or on detergent extracts of whole cells. In some cases, the difficulties associated with the short half-lives of precursors in the extramitochondrial pool have been circumvented by labelling cells under conditions where import into the mitochondrion is inhibited, e.g. in the presence of uncouplers of oxidative phosphorylation (see section 1.2.5).

More recently, the techniques which are now available for isolating and manipulating genes have been used to study the molecular features of the import process. Many nuclear genes encoding mito-chondrial polypeptides have been isolated and partially characterised. By sequencing the cloned genes, it has been possible to identify the presence of a cleavable signal sequence in the precursor molecule,

provided that the N-terminal of the mature protein is known. Molecular cloning studies have been performed largely in Saccharomyces cerevisiae, because of the suitability of this organism to genetic manipulation, and since many of the biochemical studies of mitochondrial protein import have been performed in yeast.

Since 1979, when it was reported that the nuclearly encoded α-, β-, and γ-subunits of the yeast ATPase were initially synthesised as higher M_r precursors (Maccecchini et al., 1979), many laboratories have described higher M_r precursor forms of a large number of mitochondrial polypeptides. These precursors possess M_r values which are between 500 and 10000 greater than their mature counterparts (Table 1.1). In those cases which have been directly investigated, it has been shown that the difference in M_r value results from an N-terminal polypeptide extension. It is likely that N-terminal extensions will prove to be the rule, but the possibility that modifications occur also at the C-terminus cannot be excluded.

There is no apparent correlation between the size of the N-terminal extension and the final intramitochondrial location of the mature protein. Furthermore, precursors to different subunits of a hetero-oligomeric assembly have no standard number of additional amino acids (Maccecchini et al., 1979; Lewin et al., 1980; Mihara and Blobel, 1980; Teintze et al., 1982).

A few of the mitochondrial polypeptides which are imported into internal locations are synthesised without a cleavable polypeptide extension. Among these are two inner membrane proteins; the adenine nucleotide translocase (Zimmermann and Neupert, 1980) and thermogenin (Freeman et al., 1983); a matrix protein, 2-isopropylmalate synthase (Gasser et al., 1982b; Hampsey et al., 1983), and cytochrome c, a

TABLE 1.1 Cytoplasmically synthesised mitochondrial proteins (After Hay *et al*. (1984))

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($\times 10^{-3}$)		REFERENCE
			MATURE	PRECURSOR	
Matrix	Acetoacetyl-CoA thiolase	Rat	38	41	Hay *et al*. (1984)
	Acyl-CoA dehydrogenase				
	general	Rat	39	41	"
	short-chain	Rat	36.5	41	"
	Adrenodoxin	Cattle	12	20	Nabi & Omura (1980)
	Adrenodoxin reductase	Cattle	~50	~50	"
	-Aminolevulinate synthase	Rat	45	51	Yamauchi *et al*. (1980)
		Chicken	63-65	75	Ades & Harpe (1981)
	Aspartate aminotransferase	Chicken	44.5	47	Sonderegger *et al*. (1980)
		Rat	45	47	Sakakibara *et al*. (1980)
	F_1-ATPase: α-subunit	Yeast	58	64	Maccecchini *et al*. (1979)
	β-subunit	Yeast	54	56	"
	γ-subunit	Yeast	34	40	"
	Carbamyl-phosphate synthetase	Rat	160	165	Mori *et al*. (1979)
		Frog	160	160	Campbell *et al*. (1982)
	Citrate synthase	Yeast	47	50	Böhni *et al*. (1983)
		N. crassa	45	47	Harmey & Neupert (1979)
	Enoyl-CoA hydratase	Rat	25	29.5	Hay *et al*. (1984)
	L-glutamate dehydrogenase	Rat	54	60	Mihara *et al*. (1982a)
	3-Hydroxyacyl-CoA dehydrogenase	Rat	29.5	33	Hay *et al*. (1984)
	2-Isopropylmalate synthase	Yeast	65	65	Gasser *et al*. (1982a)
	3-Ketoacyl-CoA thiolase	Rat	38	38	Hay *et al*. (1984)
	Malate dehydrogenase	Rat	37	38	Mihara *et al*. (1982a)
	Manganese superoxide dismutase	Yeast	24	26	Autor (1982)
	Methylmalonyl-CoA mutase	Rat	75	80.5	Hay *et al*. (1984)
	Ornithine aminotransferase	Rat	43	49	Mueckler *et al*. (1982)
	Ornithine transcarbamylase	Rat	36-39	39.5-43	Mori *et al*. (1981)
		Mouse	37	39.5	Hay *et al*. (1984)

TABLE 1.1 (cont'd)

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($x10^{-3}$)		REFERENCE
			MATURE	PRECURSOR	
Matrix	Phenylalanyl tRNA synthetase	Yeast			Diatewa & Stahl (1981)
	α-subunit		72	74	"
	β-subunit		57	61	"
	Propionyl-CoA carboxylase	Rat			Hay *et al*. (1984)
	α-subunit		70	74.5	"
	β-subunit		54	61.5	"
	RNA polymerase	Yeast	45	47	Lustig *et al*. (1982)
Inner Membrane	ADP/ATP carrier	*N. crassa*	32	32	Zimmermann & Neupert (1980)
		Rat	30	30	Hatalovå & Kolarov (1983)
	ATPase subunit 9	*N. crassa*	8.2	14	Michael *et al*. (1979)
	Carnitine acetyltransferase	Rat	67.5	69	Miyazawa *et al*. (1983)
	Creatine kinase	Dog	42	48	Perryman *et al*. (1983)
	Cholesterol-side-chain-cleavage cytochrome P-450	Cattle	49	54.5	DuBois *et al*. (1981)
	Cytochrome c oxidase: subunit IV	Yeast	14	17	Lewin *et al*. (1980)
	subunit V	Yeast	12.5	15	Mihara & Blobel (1980)
	subunit VI	Yeast	12.5	17-20	"
	subunit VII	Yeast	5-7.5	5-7.5	"
	subunit IV	Rat	16.5	18-19.5	Schmelzer & Heinrich (1980)
	subunit V	Rat	12.5	15.5	"
	D-β-Hydroxybutyrate dehydrogenase	Rat	32	37	Mihara *et al*. (1982a)
	Cytochrome bc_1 complex:				Côtė *et al*. (1979)
	subunit I	Yeast	44	44.5	"
	subunit II	Yeast	40	40.5	"
	subunit V	Yeast	25	27	"
	subunit VI	Yeast	17	25	"
	subunit VII	Yeast	14	14	"
	subunit VIII	Yeast	11	11	"

TABLE 1.1 (cont'd)

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($\times 10^{-3}$)		REFERENCE
			MATURE	PRECURSOR	
Inner Membrane	Cytochrome c_1	Yeast	31	37	Ohashi *et al.* (1982)
	Cytochrome bc_1 complex:	*N. crassa*			Teintze *et al.* (1982)
	subunit I	"	50	51.5	"
	subunit II	"	45	47.5	"
	subunit V	"	25	28	"
	subunit VI	"	14	14	"
	subunit VII	"	11.5	12	"
	subunit VIII	"	11.2	11.6	"
	Cytochrome c_1	"	31	38	"
Intermembrane Space	Adenylate kinase	Chicken	28	28	Watanabe & Kubo (1982)
	Cytochrome c	*N. crassa*	12	12	Korb & Neupert (1978)
		Rat	12	12	Matsuura *et al.* (1981)
	Cytochrome c peroxidase	Yeast	33.5	39.5	Maccecchini *et al.* (1979)
	Cytochrome b_2	Yeast	58	68	Daum *et al.* (1982b)
	Sulphite oxidase	Rat	55	59	Mihara *et al.* (1982a)
Outer Membrane	Monoamine oxidase	Rat	59	59	Sagara & Ito (1982)
	Porin	Yeast	29	29	Gasser & Schatz (1983)
		N. crassa	31	31	Freitag *et al.* (1982a,b)
	14000-M_r protein	Yeast	14	14	Hay *et al.* (1984)
	45000-M_r protein	Yeast	45	45	"
	70000-M_r protein	Yeast	70	70	"
	OMM-35	Rat	35	~35.5	Shore *et al.* (1981)

component of the intermembrane space (Korb and Neupert, 1978; Zimmermann *et al*., 1979). With one possible exception (Shore *et al*., 1981), all of the proteins of the mitochondrial outer membrane studied so far are synthesised without a transient polypeptide extension (Hay *et al*., 1984).

There is evidence to suggest that the extramitochondrial precursor forms of at least some mitochondrial proteins are markedly different in conformation from their intramitochondrial mature counterparts. For example, the extramitochondrial precursor of the *N. crassa* adenine nucleotide translocase binds to hydroxylapatite, whereas the mature form does not (Zimmermann and Neupert, 1980), although the primary sequence of the mature protein is identical to that of the initial translation product. Another example is seen in the case of cytochrome c; antibodies raised to apocytochrome c recognise the apoenzyme, but not the holoenzyme, and vice versa (Korb and Neupert, 1978).

Conformational differences between precursor and mature polypeptides are also suggested by intermolecular associations. The precursor to rat liver ornithine transcarbamylase sediments as a particle of 14S, much faster than the mature trimeric enzyme at 6S (Miura *et al*., 1981). Aggregates of precursors are also found for the *in vitro*-synthesised adenine nucleotide translocase (Zimmermann and Neupert, 1980), and for the α- and β-subunits of the yeast ATPase (Hay *et al*., 1984). The *in vivo*-synthesised precursor of the β-subunit (M_r = 56000) behaves as a 500000-M_r species on gel filtration. However, it is not known whether the β-subunit precursors form large homo-oligomers, aggregates with precursors of other subunits or are associated with cytosolic import factors.

1.2.3.3 FEATURES AND FUNCTIONS OF MITOCHONDRIAL SIGNAL SEQUENCES

Although precursor molecules have proved difficult to isolate in appreciable quantities, the amino acid sequences of approximately thirty cleavable mitochondrial signal sequences have been deduced by DNA sequence analysis of the corresponding cloned genes (Reid, 1985; von Heijne, 1986). These sequences do not show extensive primary sequence homology; however, most share similar overall characteristics. Signal sequences of mitochondrial matrix or inner membrane proteins are usually basic, being rich in the hydroxylated amino acids serine and threonine and in the positively-charged amino acids arginine and lysine. The latter residues occur more or less periodically along the sequence and are usually separated by three to five uncharged residues. Signal sequences are usually devoid of acidic residues.

The N-terminal extensions of proteins exposed to or present in the intermembrane space contain a long stretch of uncharged amino acids adjacent to an N-terminal region rich in basic and hydroxylated residues (Kaput *et al*., 1982; Sadler *et al*., 1984). Although proteins of the outer membrane are generally synthesised without a cleavable signal sequence (see section 1.2.3.2), the N-terminal sequence of at least one outer membrane protein (the yeast 70000-M_r outer membrane protein) resembles signal sequences of intermembrane space proteins, in that the basic N-terminus is followed by a stretch of uncharged residues (Hase *et al*., 1984). Therefore, in the remainder of this section, the term "presequence" is used to include such non-cleaved N-terminal sequences.

When mitochondrial polypeptide precursors synthesised *in vitro* are treated with processing protease isolated from the mitochondrial

matrix, the resulting mature-sized polypetides are no longer imported into isolated mitochondria (Gasser *et al.*, 1982b). This finding indicated that the cleavable extension is essential for directing precursor polypeptides into mitochondria, but did not exclude the possibility that some information is also provided by that part of the precursor molecule which is retained in the mature protein. However, characterisation of the information contained within mitochondrial presequences has been achieved recently using a combination of deletion analyses and gene fusion experiments (Hurt and van Loon, 1986; Douglas *et al.*, 1986).

Hurt *et al.* (1984) showed that the cleavable N-terminal polypeptide of the yeast cytochrome c oxidase subunit IV precursor is sufficient to direct a foreign cytosolic protein (mouse dihydrofolate reductase; DHFR) into the matrix of yeast mitochondria *in vitro*. Similar conclusions have been reported for the targetting of cytosolic proteins by the transient polypeptides of other imported matrix proteins, including human ornithine transcarbamylase (Horwich *et al.*, 1985), yeast 5-aminolevulinate synthase (Keng *et al.*, 1986) and yeast alcohol dehydrogenase III (van Loon *et al.*, 1986). Conversely, imported mitochondrial proteins lacking all or even some of their own presequences are not imported into mitochondria (Hurt *et al.*, 1985; van Loon and Young, 1986). Mitochondrial presequences thus contain information which functions in "intracellular targetting".

The gene fusion experiments referred to above showed that a given cleavable presequence could be fused to more than one protein and effect its import into mitochondria. This strongly suggests that; 1) these N-terminal sequences act, and probably fold, independently from the mature protein; 2) the mature segment of the precursor polypeptide

need not participate in targetting of a protein to the mitochondrion.

Gene fusion experiments have revealed also that the presequences of mitochondrial polypeptide precursors contain information for "intramitochondrial sorting" as well as for intracellular targetting. Van Loon et al. (1986) have shown that the first 32 amino acids of the cleavable presequence of cytochrome c_1 (an inner membrane protein protruding into the intermembrane space) targets mouse DHFR to the mitochondrial matrix. However, inclusion of the remainder of the complete presequence, which contains a stretch of 19 uncharged residues, results in the appearance of DHFR in the intermembrane space; unlike cytochrome c_1, DHFR does not reinsert into the inner membrane via its C-terminal end. Thus, the membrane-anchoring sectors within presequences of intermembrane space proteins appear to have a crucial role in preventing transport of the entire precursor into the matrix.

Additional evidence for the existence of multiple targetting signals within mitochondrial presequences comes from studies on the yeast 70000-M_r outer membrane protein. Although this protein is synthesised without a cleavable polypeptide extension, its 12 N-terminal residues can direct an attached protein to the yeast mitochondrial matrix in vivo and in vitro (Hurt et al., 1985b). In contrast, the N-terminal 41 residue segment of the 70000-M_r outer membrane protein (which contains the putative outer membrane anchor sequence) directs an attached foreign protein to the mitochondrial outer membrane (Hase et al., 1984).

The information obtained from gene fusion experiments has prompted the formation of a model for intracellular targetting and intra-mitochondrial sorting of imported mitochondrial proteins (Hurt and van

Loon, 1986). This model proposes that N-terminal presequences of imported mitochondrial proteins can consist of a relatively simple, linear array of short matrix-targetting domains, stop-tranfer domains and cleavage sites. A matrix-targetting domain (which consists of a periodic array of basic amino acids) directs an attached protein specifically to mitochondria and across both mitochondrial membranes. If a matrix-targetting domain is followed by a stop-transport domain (which contains a long, uninterrupted stretch of uncharged residues followed by charged residues), protein transport across the mitochondrial inner and/or outer membrane is prevented; the protein becomes a component of the outer membrane or becomes a protein exposed to or released into the intermembrane space. Cleavage sites may be present for proteolytic removal of all or part of the presequence.

The localisation of proteins to the mitochondrial outer membrane (rather than the inner membrane) may be due to a greater proximity of matrix-targetting and membrane-anchoring sequences in outer membrane proteins, such that matrix penetration of the N-terminus is prevented. However, differential membrane specificity of the two anchoring sequences cannot be ruled out at this stage.

Clearly, there are exceptions to the above model. Examples include the import of cytochrome c, which is targetted to the intermembrane space without a cleavable polypeptide extension (Hennig and Neupert, 1981), and of subunit VI of the cytochrome bc_1 complex, which is synthesised with an acidic N-terminal extension (van Loon *et al.*, 1984).

1.2.4 BINDING OF PRECURSORS TO THE MITOCHONDRIAL OUTER MEMBRANE

The second step in the biogenetic pathway of nuclearly encoded mitochondrial proteins requires the specific interaction of precursor polypeptides with the cytoplasmic face of the mitochondrial outer membrane. Since precursor molecules have been shown to possess specific "mitochondrial targetting" signals, one would predict the existence of receptor molecules on the mitochondrial surface which recognise these signals. Indeed, the available evidence strongly implicates the participation of such receptor molecules in the import process.

The most extensive evidence for a mitochondrial import receptor comes from studies by Neupert and coworkers on the import of cytochrome c into *N. crassa* mitochondria. These studies have exploited the fact that relatively large amounts of apocytochrome c can be prepared chemically. Although the precursor form of cytochrome c does not contain a cleavable polypeptide extension, its maturation involves covalent attachment of haem. Thus, the cytochrome precursor is equivalent to apocytochrome c which has been prepared by chemical removal of the haem group from the holoenzyme.

The apocytochrome c receptor has been detected by its ability to bind radiolabelled precursor in an *in vitro* assay. The precursor was synthesised in a cell-free translation system in the presence of [^{35}S]methionine, then incubated with isolated mitochondria. Under suitable conditions, the extramitochondrial apocytochrome c was converted to intramitochondrial holocytochrome c (Hennig and Neupert, 1981). Only a small fraction of the apocytochrome c was recovered with reisolated mitochondria. To study binding in the absence of net

transport, deuterohaemin (an inhibitor of the haem attachment step) was added to the mitochondria before adding precursor proteins. Under these conditions, about half of the apocytochrome c was found associated with mitochondria which was sensitive to externally-added protease. Thus, in the presence of deuterohaemin, apocytochrome c appears to accumulate on the outer surface of the mitochondrion.

The sites to which apocytochrome c is bound are apparently specific and relevant to the import pathway. Firstly, apocytochrome c which is very tightly bound to the mitochondria can be released by addition of excess chemically prepared apocytochrome but not by holocytochrome c, a finding indicating reversibility as well as specificity of the binding reaction. Secondly, when the inhibition of haem attachment is reversed with protohaemin, apocytochrome c bound to the mitochondrial surface is transported into mitochondria and converted to the holoenzyme.

The existence of more than one type of import receptor is suggested by the finding that the binding sites involved in the import of apocytochrome c are not required for entry of other cytoplasmically-synthesised polypeptides into mitochondria. Whereas the appearance of intramitochondrial, radiolabelled holocytochrome c was almost completely blocked using unlabelled apocytochrome c, the import of the adenine nucleotide translocase and of the ATPase subunit 9 was unaffected under the same conditions (Zimmermann et al., 1981). Moreover, when the import of the adenine nucleotide translocase was inhibited by elastase treatment of isolated N. crassa mitochondria, import of the ATPase subunit 9 was unaffected by the same level of protease (Zwizinski et al., 1984). Taken together, these data suggest that at least three types of binding site are involved in the import

of precursor polypeptides into mitochondria.

The precursor binding activity appears to involve polypeptide components of the mitochondrial outer membrane. This is inferred from the findings that mild protease treatment of isolated mitochondria inhibits their ability to bind and import precursors (Gasser et al., 1982b; Argan et al., 1983; Zwizinski et al., 1984), and that isolated, right side-out outer membrane vesicles are capable of binding specifically precursors, in a manner which is rapid and apparently of high affinity. Such binding was shown to be exclusive to the cytoplasmic surface of the mitochondrial outer membrane, being absent from preparations of inner membrane or microsomes (Riezman et al., 1983b). As with isolated mitochondria, the precursor binding capacity of outer membrane vesicles is sensitive to mild protease treatment.

It is highly unlikely that each species of imported precursor has its own receptor on the mitochondrial surface. In recent studies, purified 3-ketoacyl-CoA thiolase was found to inhibit the in vitro import of three other mitochondrial polypeptide precursors into rat liver mitochondria (Miura et al., 1985). This result indicated that a common mitochondrial component participates in the matrix delivery of these proteins in rat liver. In addition, analysis of the polypeptide composition of the mitochondrial outer membrane by SDS/polyacrylamide gel electrophoresis reveals a relatively small number of protein bands (Riezman et al., 1983a; Shore et al., 1981).

There is evidence to suggest that the binding of precursor molecules to the surface of mitochondria is mediated by soluble cytoplasmic components. In an in vitro import assay, import of pre-ornithine transcarbamylase into rat liver mitochondria requires a factor contained in rabbit reticulocyte lysate (Argan et al., 1983;

Miura *et al.*, 1983). Similarly, the *in vitro* import of pre-ATPase -subunit into yeast mitochondria requires a factor present in yeast cytosol or rabbit reticulocyte lysate (Ohta & Schatz, 1984). In both cases, the cytosolic factor exhibited properties typical of a protein. The yeast cytosolic factor was estimated by gel filtration to have an M_r value of 40000 (Ohta & Schatz, 1984).

More recent studies indicate that a protein import factor associates with the ornithine transcarbamylase precursor before binding of the factor-precursor complex to mitochondria (Argan and Shore, 1985). The import factor, however, does not bind to mitochondria in the absence of precursor. Thus, the association of precursor with the cytosolic factor may be required for the binding to components on the mitochondrial surface.

It has also been suggested that a cytoplasmic RNA moiety is required for import of precursor polypeptides into mitochondria (Firgaira *et al.*, 1984). This suggestion was based on the finding that posttranslational addition of ribonuclease to a cell-free translation system inhibited subsequent import of a range of polypeptide precursors into the rat liver mitochondrial matrix. However, a subsequent report (Burns and Lewin, 1986) provides evidence which indicates that the inhibition of import by ribonuclease is an effect of degraded ribosomes in the cell-free translation mixture, rather than a requirement of the import process for RNA.

1.2.5 TRANSLOCATION AND ENERGY DEPENDENCE

Once bound to the cytoplasmic face of the mitochondrial outer membrane, a cytoplasmically-synthesised precursor polypeptide inserts

into this lipid bilayer to become a component of the outer membrane, or is translocated into the mitochondrial interior to eventually become a component of the intermembrane space, inner membrane or matrix. Although very little is known about the mechanism of protein translocation, the existence of a number of different import pathways has been established.

Translocation of precursor polypeptides into or across the mitochondrial inner membrane is energy dependent. This was initially indicated by Nelson and Schatz (1979), who showed that in pulse-labelled yeast spheroplasts, the import of precursors of the α-, β- and γ- subunits of the F_1 ATPase, and of two subunits of the cytochrome bc_1 complex was blocked in the presence of CCCP, an uncoupler of oxidative phosphorylation, or by depleting the level of matrix ATP in non-respiring yeast strains. Subsequently, it was shown that the import of the adenine nucleotide translocase was blocked by CCCP (Zimmermann et al., 1981). From these observations, however, it was not possible to distinguish rigorously between a requirement for matrix ATP or for an electrochemical potential across the inner membrane, since the addition of uncoupler renders the inner membrane permeable to protons and simultaneously stimulates the hydrolysis of ATP by the F_1 ATPase (Heytler, 1979). However, Nelson and Schatz proposed that matrix ATP was probably the immediate energy donor for import, since respiration deficient (ρ^-) yeast strains, which cannot form an appreciable membrane potential, still import proteins from the cytosol.

Definitive evidence to the contrary has since been obtained by investigating in vitro import in the presence of respiratory chain inhibitors, ionophores or uncouplers of oxidative phosphorylation.

Gasser *et al*. (1982a) used an *in vitro* import system in which precursor-containing reticulocyte lysate was gel-filtered to remove energy sources, and isolated mitochondria were treated with cyanide to inhibit respiration. The cyanide-treated mitochondria were incubated with the gel-filtered lysate in the presence of ATP. Under these conditions, a large fraction of the ATPase β-subunit precursor was imported into mitochondria. This import was entirely dependent on added ATP and could be blocked by addition of either carboxy-atractyloside, an inhibitor of the adenine nucleotide translocase or oligomycin, an inhibitor of the proton-translocating ATPase.

The inhibition of protein import by these two compounds indicates that added ATP must enter the mitochondrion and be hydrolysed by the F_1F_0-ATPase for translocation to occur. Since oligomycin inhibits hydrolysis of ATP by the ATPase, it should increase the concentration of ATP in the mitochondrial matrix. Therefore, the observation that oligomycin blocks protein import suggested that matrix ATP is not the direct source of energy for translocation. This was confirmed by demonstrating that the inhibitory effect of oligomycin could be overcome by presenting mitochondria with a substrate for respiration, thus restoring a transmembrane electrochemical potential. Therefore, the ability of mitochondria to import proteins correlated with conditions where the electrochemical potential gradient would be expected to be relatively large, regardless of the matrix ATP concentration.

Schleyer *et al*. (1982) studied the energy requirement associated with import of the ATPase subunit 9 precursor into *N. crassa* mitochondria. Import was inhibited using a combination of oligomycin and antimycin A, an inhibitor of electron transfer through the

cytochrome bc_1 complex. The translocation activity could be restored upon addition of ascorbate and tetramethylphenylenediamine (TMPD), thereby allowing reduction of cytochrome c and the subsequent regeneration of a proton electrochemical potential gradient by the activity of cytochrome c oxidase. The matrix ATP concentration should be unaffected by the addition of ascorbate and TMPD, yet import proceeded efficiently, again implicating a requirement for an electrochemical potential gradient across the mitochondrial inner membrane.

The electrochemical potential gradient ($\Delta\tilde{\mu}H^+$) is the sum of a proton gradient (ΔpH) and the potential generated from the separation of charged species across the inner membrane ($\Delta\psi$). To investigate the relative importance of ΔpH and $\Delta\psi$ in protein translocation into or across the mitochondrial inner membrane, Pfanner and Neupert (1985) developed a buffer system in which _in vitro_ import of the adenine nucleotide translocase from the receptor-bound state was monitored, while ΔpH and $\Delta\psi$ were manipulated. Using this buffer system, it was found that import could be driven by a valinomycin-induced potassium diffusion potential, whereas imposition of a ΔpH did not stimulate import. In addition, the protonophore CCCP did not abolish the import driven by a potassium diffusion potential. These results suggest that the membrane potential ($\Delta\psi$) itself is the required energy form for import, rather than a proton gradient.

The import of proteins into the mitochondrial outer membrane occurs in an energy-independent manner (Gasser and Schatz, 1983; Freitag _et al_., 1982). Similarly, the import of cytochrome c into the intermembrane space does not require a transmembrane electrochemical potential gradient (Zimmermann _et al_., 1981), presumably because the

mitochondrial inner membrane is not directly involved in the import of this protein. Surprisingly, the import of other soluble intermembrane space components such as cytochrome b_2 and cytochrome c peroxidase is energy dependent (Reid *et al.*, 1982). However, these findings are rationalised by evidence which indicates that the maturation of these two proteins involves partial translocation of their respective precursors across the mitochondrial inner membrane (Reid *et al.*, 1982; see also section 1.2.6).

Several findings indicate that translocation rather than proteolytic processing of precursors is the energy dependent step in protein import. Firstly, the import into the inner membrane or matrix of precursors which are synthesised without a cleavable polypeptide extension (e.g. isopropylmalate synthase or the adenine nucleotide translocase) requires energy, although no proteolytic processing is involved (Gasser *et al.*, 1982b; Hampsey *et al.*, 1982; Schleyer *et al.*, 1982). Secondly, processing is still catalysed by a partially purified matrix protease (see section 1.2.6). Thirdly, the transport and processing of cytochrome c peroxidase are temporally separated; the initial transport step is blocked by CCCP, whereas subsequent processing is not (Reid *et al.*, 1982).

Schleyer and Neupert (1985) have monitored the intermediate reactions during protein import into mitochondria. Translocation intermediates were trapped within the mitochondrial membranes by inhibiting translocation either at low temperature, or by pre-binding specific antibodies to the mature part of precursor proteins. Under such conditions, the N-terminal presequence was translocated into the matrix and removed by the matrix processing protease, while the major part of the precursor molecule was still outside the mitochondrial

outer membrane. These data suggest that at least some mitochondrial precursors may cross the outer and inner membranes through regions where the two membranes are in close proximity, in agreement with earlier electron microscopic evidence of mitochondrially-bound ribosomes which are preferentially present at sites where outer and inner membrane come into close contact (Kellems et al., 1975).

The precise role played by the membrane potential in precursor translocation remains to be determined. However, Schleyer and Neupert (1985) have shown that an energised inner membrane is required only for the initial translocation of the N-terminal portion (containing the positively-charged presequence), but not for that of the remainder of the precursor molecule. In addition, the insertion of synthetic presequences into lipid vesicles is facilitated by a valinomycin-induced diffusion potential across the liposome membrane but only if the inside of the vesicle is negatively charged, i.e. of the same polarity as that found in intact respiring mitochondria (Roise et al., 1986). Thus, the membrane potential may mediate translocation by exerting an electrophoretic effect on positively-charged N-terminal sequences.

1.2.6 PROCESSING OF PRECURSOR MOLECULES

During or shortly after their transmembrane passage, most mitochondrial precursor polypeptides undergo processing, thereby generating the mature form of the protein. Processing most commonly involves the proteolytic removal of the transient N-terminal extension from the precursor via one or two cleavage events. The first of these cleavages is catalysed by a soluble component of the mitochondrial

matrix in yeast, rat and *Zea mays* (Böhni *et al.*, 1980, 1983; McAda and Douglas, 1982; Mori *et al.*, 1980; Miura *et al.*, 1982). Its activity is maximal at neutral pH, is insensitive to serine protease inhibitors, but is inhibited by chelators of divalent metal ions such as 1,10-phenanthroline, EDTA, and GTP. This inhibition could be at least partially reversed by addition of an excess of Zn^{2+} or Co^{2+} (Böhni *et al.*, 1983; Conboy *et al.*, 1982) or Mn^{2+} (McAda and Douglas, 1982). It is not known which of these cations is normally present in the active enzyme.

The yeast matrix protease has been partially purified (McAda and Douglas, 1982; Böhni *et al.*, 1983). Böhni *et al.* (1983) demonstrated that this enzyme is responsible for the cleavage of several higher M_r precursors of imported mitochondrial polypeptides; it may in fact cleave all mitochondrial precursors with N-terminal extensions. In this respect the enzyme may be considered to have a broad specificity. In contrast, the yeast matrix protease is highly specific for native precursors, being inactive towards denatured precursors and non-mitochondrial proteins. This conformational requirement indicates that the protease does not simply recognise a particular amino acid sequence, but rather some three-dimensional domain of the precursor. Since the partially purified protease cleaves *in vitro*-synthesised precursors in the absence of mitochondria, it appears that the conformation of at least the N-terminal precursor regions are similar in solution and during (or soon after) translocation into the matrix.

The matrix protease appears to be an endoprotease since partially processed intermediates are not normally observed. Confirmation of this situation requires detection of the intact N-terminal peptide after processing, but this has not yet been achieved.

The processing of some proteins of the intermembrane space involves the matrix protease described above; a situation which would not be predicted from the submitochondrial location of such proteins. Cytochrome b_2 (a soluble intermembrane space component) and cytochrome c_1 (which is attached to the inner membrane with its bulk protruding into the intermembrane space) have been shown clearly to be processed by two distinct proteolytic cleavage events. It is highly likely that another soluble intermembrane space protein, cytochrome c peroxidase also follows the same route (Teintze *et al*., 1982; Gasser *et al*., 1982b; Ohashi *et al*., 1982; Reid *et al*., 1982; Daum *et al*., 1982a,b).

The suggested pathway for the maturation of these three cytochromes is as follows. The first of two cleavages is catalysed by the matrix protease and yields intermediate forms of each protein which migrate electrophoretically between the mature and precursor forms. These intermediates are firmly bound to the inner membrane and protrude into the intermembrane space. The second cleavage, which yields the mature-sized polypeptides, occurs on the outer surface of the inner membrane. The mature forms of cytochrome b_2 and cytochrome c peroxidase are released as soluble components of the intermembrane space, whereas mature cytochrome c_1 remains anchored in the inner membrane.

In yeast, at least, the enzyme responsible for the second cleavage is different from the matrix protease, since it is resistant to chelating agents and highly sensitive to detergent (Daum *et al*., 1982b).

Intermediate forms of yeast cytochromes b_2 and c_1 can be generated by incubating *in vitro*-synthesised precursors of these two proteins with partially-purified matrix protease (Böhni *et al*., 1983).

Such intermediate forms can also be detected *in vivo*. Intact yeast cells or spheroplasts were pulse-labelled while their mitochondria were uncoupled, so that the precursor forms of cytochromes b_2 and c_1 accumulated in the cytosol (Gasser *et al*., 1982b; Ohashi *et al*., 1982; Reid *et al*., 1982). Following a chase period when the block on import was reversed, the intermediate forms of the cytochromes, and subsequently their mature forms appeared.

The cytochrome c peroxidase precursor is cleaved to an intermediate form by the partially-purified matrix protease (Gasser *et al*., 1982b), and its processing *in vivo* is blocked by 1,10-phenanthroline (Reid *et al*., 1982), suggesting a role for the matrix protease. However, no cytochrome c peroxidase intermediate has been detected *in vivo*. Cleavage of the cytochrome c peroxidase precursor by the matrix protease is relatively slow and therefore may be rate limiting. If this were the case, the intermediate would not then be detected for kinetic reasons.

During its maturation, cytochrome c_1 undergoes covalent attachment of haem. Ohashi *et al*. (1982) have investigated when this reaction occurs in relation to the two proteolytic cleavages. This was achieved using a haem-deficient yeast mutant which lacked 5-aminolevulinate synthase. When this mutant was pulse-labelled in the absence of haem precursors, it was found that the intermediate form of cytochrome c_1 was accumulated. When the pulse-labelled cells were chased in the presence of 5-aminolevulinate, the accumulated intermediate form was converted to mature cytochrome c_1. Thus, the second processing step is dependent on the availability of haem, presumably because haem attachment to the intermediate renders it sensitive to the second protease.

Besides proteolytic cleavage and the haem attachment described above, other forms of processing of mitochondrial precursors are known. These include covalent attachment of flavin nucleotides, incorporation of cofactors such as TPP or pyridoxal phosphate, the methylation of amino-acyl side chains, and acetylation of the mature amino-terminus (Hay *et al.*, 1984). Mature cytochrome c contains ε-aminotrimethyllysine in *N. crassa* (De Lange *et al.*, 1970) and wheat germ (De Lange *et al.*, 1969) and its N-terminus is acetylated in many mammals (Margoliash, 1966). On the whole, the roles played by the enzymes effecting such modifications are poorly defined, although it is clear that non-proteolytic processing (as in the aforementioned case of cytochrome c_1) can be essential for generation of a functional protein.

1.2.7 ASSEMBLY OF IMPORTED PROTEINS INTO FUNCTIONAL UNITS

The final stage in the biogenetic pathway of an imported mitochondrial polypeptide is the acquisition of a functional conformation in the correct submitochondrial location. In many cases, this involves specific interactions with other homologous or heterologous subunits. Biogenesis of four of the respiratory complexes of the inner membrane is especially complicated, since their assembly entails physical association of nuclear and mitochondrial gene products. In comparison with the preceding steps in the pathway, the terminal assembly event is least well understood, largely because of a lack of adequate methodology for the investigation of such intermolecular interactions.

To date, three main criteria have been used in the assessment of whether polypeptides imported into mitochondria *in vitro* become

assembled in a manner which is representative of that occurring in living cells. These are: 1) the imported protein is shown to have reached its correct submitochondrial location; 2) it should be associated with the appropriate homologous or heterologous subunits in a structure resembling the native one; and 3) the assembled subunits should exhibit biological activity.

Subfractionation of isolated yeast mitochondria after import of *in vitro*-synthesised precursors has shown that imported cytochrome b_2 (an intermembrane space protein) and isopropylmalate synthase (a matrix component) are recovered predominantly in their correct sub-mitochondrial fractions. However, a disproportionately large amount of radiolabelled, imported polypeptides was isolated with the mitochondrial membranes. The membrane-bound forms may represent intermediate steps on the maturation pathways of these proteins (Gasser *et al.*, 1982b). On the other hand, two imported inner membrane proteins were found exclusively in the membrane fraction.

Proper orientation within a membrane has been inferred for two imported mitochondrial proteins since they share a physical property with the corresponding mature counterpart. The imported form of the outer membrane porin in yeast (Gasser and Schatz, 1983; Mihara *et al.*, 1982) and in *N. crassa* (Freitag *et al.*, 1982) resembles mature porin in its high resistance to proteolysis. Also, the *N. crassa* adenine nucleotide translocase behaves like the authentic mature protein in that it does not bind to hydroxylapatite in the presence of carboxy-atractyloside (Schleyer and Neupert, 1984).

Evidence for correct homo-oligomer formation by imported translation products has been obtained in the case of ornithine transcarbamylase. Kalousek *et al.* (1984) showed that the imported and

processed form of *in vitro*-synthesised ornithine transcarbamylase, but not the precursor, binds to a ligand affinity column which recognises native ornithine transcarbamylase. Furthermore, a significant portion of radiolabelled subunits which were eluted from the affinity matrix comigrated with the native, trimeric enzyme on a gel filtration column.

No such binding to the affinity matrix was observed for the mature-sized monomer or dimer. Moreover, processing by a mitochondrial matrix fraction failed to yield trimeric enzyme, despite producing ample amounts of mature-sized monomer. These data indicate that trimeric ornithine transcarbamylase composed of mature-sized subunits can be assembled *in vitro* by intact mitochondria following translocation and proteolytic processing.

It has been suggested that *in vitro* import of rat carbamyl phosphate synthase (Campbell *et al*., 1982) and yeast phenylalanyl tRNA synthase (Diatewa and Stahl, 1981) leads to new enzyme activity. Further experimentation is required to clarify this point, the main problems being to find a system with low background enzymic activity and to import sufficient precursors to generate detectable activity.

Finally, Lewin and Norman (1983) have provided strong evidence for the proper assembly of newly imported α-, β- and γ- subunits of the yeast F_1 ATPase. Isolated yeast mitochondria were incubated with radiolabelled, *in vitro*-synthesised precursors, and newly-assembled F_1 ATPase was detected by autoradiography of the isolated enzyme. Incorporation of labelled subunits into the enzyme did not occur in the presence of uncouplers or a divalent metal ion chelator, nor did it occur when submitochondrial particles were incubated with mature-

sized *in vitro*-synthesised subunits. Furthermore, labelled subunit incorporation was not competed out when excess unlabelled subunits were added after import of radiolabelled polypeptides had taken place. These findings are indicative of *de novo* assembly of F_1 ATPase domains rather than exchange of labelled subunits with unlabelled subunits of F_1 on the mitochondrial membrane.

1.2.8 UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

Since the initial description of posttranslationally-imported higher M_r mitochondrial polypeptide precursors (Maccecchini *et al.*, 1979), the volume of literature pertaining to mitochondrial protein import has expanded at a dramatic rate. Major advances in the field include the detection of putative import receptors, elucidation of the nature of the energy dependence of import, discovery of different import pathways and, more recently, the application of recombinant DNA technology to examine the signalling information present in mitochondrial presequences.

Despite this progress, there are still considerable gaps in our knowledge of mitochondrial biogenesis. The mechanisms which coordinate the synthesis and assembly of nuclear and mitochondrial gene products remain obscure. Furthermore, little is known about the molecules which constitute the import machinery. Indeed, the putative outer membrane receptor molecules have not yet been isolated and shown to exhibit *bona fide* receptor-like activity.

A recent report by Hurt *et al.* (1986) has shown that the cleavable presequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria, albeit with relatively

low efficiency. This finding raises the intriguing question of how cytoplasmically-synthesised polypeptides are sorted between mitochondria and chloroplasts in plant cells.

Future research into mitochondrial biogenesis is likely to place considerable emphasis on a genetic approach, especially in yeast. Such an approach will allow further analysis of the sequence requirements for precursor targetting and submitochondrial localisation, and also the isolation of mutants which are defective in mitochondrial assembly.

Yaffe *et al*. (1985) have already described a temperature sensitive yeast mutant (mas 1) which is deficient in a matrix-located processing protease activity. This mutation results in arrest of growth and accumulation of the precursor forms of imported mitochondrial polypeptides. Restoration of temperature resistant growth to mas 1 mutant cells can be achieved using isolated fragments of yeast genomic DNA. Thus, analysis of the cloned gene will determine if MAS 1 is the structural gene for the matrix protein and allow further biochemical and genetic studies. By isolating and characterising a large number of mas mutants it should be possible to identify most of the components involved in mitochondrial protein import.

1.3 MULTIENZYME COMPLEXES

A consequence of the high degree of internal organisation found in eukaryotic cells is the existence of membrane-bounded intracellular compartments, which are physically and biochemically distinct. However, the compartmentation of cellular metabolism can be regarded

as occurring at other levels of cellular organisation, via the existence of "microcompartments" (Hess, 1980). The smallest metabolically significant compartments are the active sites of enzymes. These are in the Ångstrom range and serve to concentrate and sequester substrates and cofactors. Such compartments are formed most simply by the tertiary structure of a single polypeptide chain.

The next highest level of microcompartmentation is exhibited by oligomeric and multifunctional enzymes and multienzyme complexes. Both homo- and hetero-oligomeric proteins probably arose because they were energetically more stable than the monomeric state. In addition, the aggregated state affords the establishment of cooperative interactions between active sites, or between regulatory and catalytic subunits. These interactions form the basis for the allosteric regulation of key steps in cellular metabolism. In contrast to oligomeric enzymes, which catalyse a single reaction, multifunctional enzymes or multienzyme complexes catalyse two or more steps in a metabolic sequence. As a result of their multiple active sites, the latter two types of protein are effective in channelling products of one reaction which are then used as substrates for the next reaction.

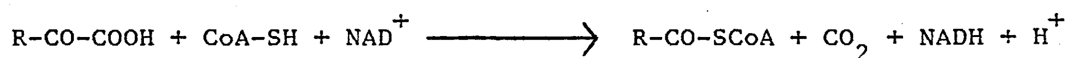
The next highest level of microcompartmentation is found in extremely sophisticated supramolecular structures such as ribosomes or the mitochondrial electron transport chain. The former represents a series of catalysts organised in an aggregate which permits the sequestration of substrates (amino acids), cofactors (ATP and GTP) and reaction products (elongating polypeptide chains) away from the bulk phase of the cytosol; the latter catalyses the transfer of electrons from NADH or $FADH_2$ to oxygen. Once electrons enter this chain they are effectively sequestered away from the aqueous environment on

either side of the mitochondrial inner membrane until they emerge from cytochrome c oxidase and combine with protons and oxygen to form water.

A number of multienzyme complexes have been studied and isolated from both prokaryotic and eukaryotic sources. These include the fatty acid synthase (Lynen, 1972) and 2-oxoacid dehydrogenase complexes (Reed, 1974; Perham, 1975) and the multienzyme complexes involved in aromatic amino acid biosynthesis (Yanofsky and Crawford, 1972; Lumsden and Coggins, 1977), which are composed of 2-6 enzymes and have M_r values ranging from approx. 150000 to several million. The 2-oxoacid dehydrogenase complexes, particularly that responsible for the oxidation of branched chain 2-oxoacids, are described in greater detail in sections 1.4-1.7.

1.4 2-OXOACID DEHYDROGENASE COMPLEXES

The overall reaction catalysed by the 2-oxoacid dehydrogenase complexes can be represented as:



$$\text{R-CO-COOH} + \text{CoA-SH} + \text{NAD}^+ \longrightarrow \text{R-CO-SCoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

where R represents the alkyl group of a 2-oxoacid (see below). These dehydrogenases consist of a family of three related multi-enzyme complexes, which, in eukaryotic cells, are located within the mitochondrion, probably loosely associated with the matrix side of the inner membrane. Each of the three complexes occupies a key position in intermediary metabolism, as illustrated in Fig.1.4.

The 2-oxoglutarate dehydrogenase complex (OGDC) is a tricarboxylic

Fig.1.4 Metabolic Roles Of The 2-Oxoacid Dehydrogenase Complexes (after Yeaman, 1986).

1, transamination;

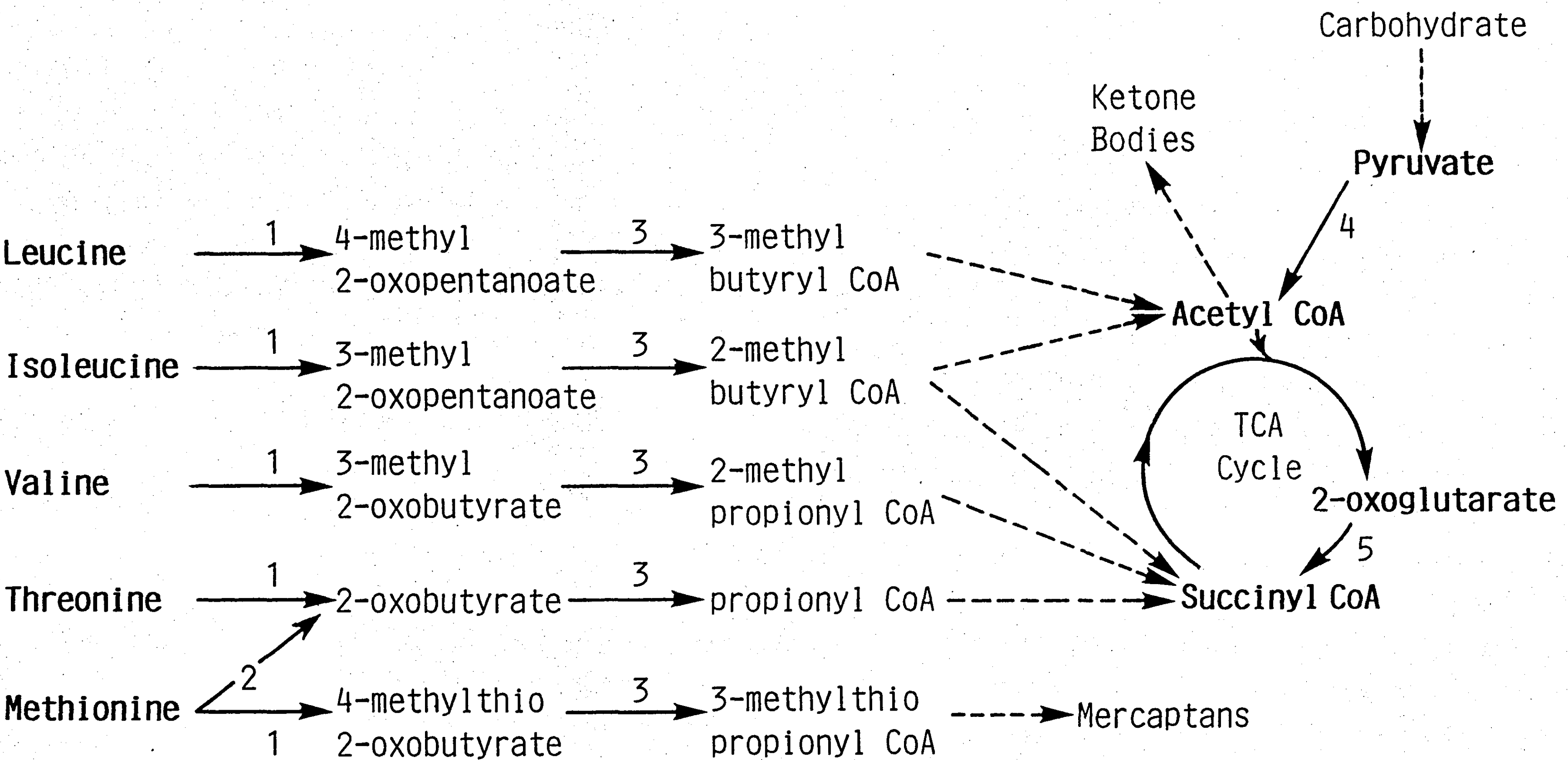
2, trans-sulphuration;

3, branched chain 2-oxoacid dehydrogenase;

4, pyruvate dehydrogenase;

5, 2-oxoglutarate dehydrogenase.

The broken lines indicate that several reaction steps are involved.



Carbohydrate
Ketone Bodies
Pyruvate
4
Leucine
1
4-methyl 2-oxopentanoate
3
3-methyl butyryl CoA
Acetyl CoA
Isoleucine
3-methyl 2-oxopentanoate
2-methyl butyryl CoA
TCA Cycle
Valine
3-methyl 2-oxobutyrate
2-methyl propionyl CoA
2-oxoglutarate
5
Threonine
2-oxobutyrate
propionyl CoA
Succinyl CoA
Methionine
2
4-methylthio 2-oxobutyrate
3-methylthio propionyl CoA
Mercaptans

acid enzyme, whilst pyruvate dehydrogenase complex (PDH) catalyses the committed reaction in the catabolism of carbohydrate. The branched chain 2-oxoacid dehydrogenase (BCOAD) complex catalyses an irreversible step in the oxidation of the branched chain amino acids leucine, isoleucine and valine. The BCOAD complex may also be involved in the catabolism of methionine and threonine, since it is reported that 2-oxobutyrate (Pettit et al., 1978) and 4-methylthio-2-oxobutyrate (Jones and Yeaman, 1986) can also be used as substrates by this complex (see Fig.1.5).

Although the three complexes are structurally and functionally distinct, they can each be isolated as a functional high-M_r assembly which contains multiple copies of three major component enzymes; E1, a substrate specific, TPP-requiring 2-oxoacid dehydrogenase; E2, a dihydrolipoamide acyltransferase which is specific for each complex and contains covalently bound lipoic acid, and dihydrolipoamide dehydrogenase (E3), a flavoprotein which, in virtually all organisms studied so far, is a common component of the three multienzyme complexes.

Deficiencies in the activities of the 2-oxoacid dehydrogenase complexes lead to pathological states, including various forms of metabolic acidosis. PDH deficiency is manifested by lactic acidaemia and BCOAD complex deficiency results in branched chain 2-oxoacidaemia or maple syrup urine disease (MSUD). Two structural bases for the latter inborn error of metabolism have been identified. A vitamin responsive form of MSUD has been described, where administration of high levels of thiamine to affected patients leads to decreased urinary output of branched chain 2-oxoacids and increased BCOAD complex activity in peripheral leukocytes (Fernhoff et al., 1981).

This finding suggests that a mutant E1 component is responsible for at least some cases of the vitamin responsive form of the disease. In addition, Danner _et al_. (1985) have shown that the BCOAD complex in fibroblast mitochondria from a thiamine nonresponsive MSUD patient lacks specifically the E2 component.

In the case of the mammalian PDH, an additional polypeptide component, termed component or protein X has recently been identified (De Marcucci and Lindsay, 1985; Jilka _et al_., 1986). This polypeptide has been shown to be similar to E2, in that it undergoes acetylation via a lipoic acid moiety (Hodgson _et al_., 1986; De Marcucci _et al_., 1986a), although a precise role for X has yet to be established. To date, no evidence has been obtained for the existence of an equivalent component in the OGDC or BCOAD complexes.

Studies on the quaternary structure of the complexes have revealed that in both bacteria and mammals, the E2 component forms a symmetrical core around which are arranged the E1 and E3 components. However, the stoicheiometry and arrangement of the components of the three mammalian complexes differs in each case (see section 1.5).

1.5 STRUCTURE AND FUNCTION OF THE BCOAD COMPLEX

The BCOAD complex was originally purified from bovine kidney, and was found to consist of a 52000-M_r component and an E1 component composed of two subunits, termed α and β, which have M_r values of 46000 and 35000, respectively (Pettit _et al_., 1978). The E3 component was found to be absent from the purified complex. Subsequently, BCOAD complex has been purified from bovine liver (Danner _et al_., 1979; Heffelfinger _et al_., 1983), rabbit liver (Paxton and Harris, 1982),

rat kidney (Odessey, 1982) and Pseudomonas putida (Sokatch et al., 1981). The E2, E1α and E1β components from each of these sources exhibit highly similar, or identical M_r values to the corresponding bovine kidney subunits. The preparations of bovine kidney BCOAD complex described by Danner et al. (1979) and Heffelfinger et al. (1983) are distinct in that they contain endogenous E3.

The three major component enzymes of the BCOAD complex act in sequence to catalyse the overall oxidative decarboxylation reaction, as illustrated in Fig.1.5. E1 catalyses the decarboxylation of the 2-oxoacid, with formation of a hydroxyacyl-TPP derivative (reaction 1). E1 then catalyses the reductive acylation of the covalently bound lipoic acid cofactor of the dihydrolipoamide acyltransferase (reaction 2). The acyl group is then transferred by E2 to the CoA acceptor, leaving the lipoic acid in the reduced state (reaction 3). E3 catalyses the reoxidation of the dihydrolipoyl moiety, with NAD^+ as the ultimate electron acceptor (reactions 4 and 5).

The mammalian BCOAD complex is organised about an oligomeric core of E2 polypeptides; this is apparently composed of 24 subunits which are arranged in groups of three about the 8 vertices of a cube. The molecules of E1 are distributed on the surface of the E2 cube, attached by non-covalent bonds (Pettit et al., 1978). The results of limited tryptic digestion of bovine liver BCOAD complex suggest that the branched chain E2 component may consist of two domains; one which forms the core aggregate and contains the transacylase active site, and a second which contains the lipoyl moiety. Chuang et al. (1985) have shown that the trypsinised E2 maintains a highly assembled core structure which contains two related lipoate-free tryptic fragments. These are a 22000-M_r species and a 26000-M_r species from which the

Fig.1.5 Sequence Of Reactions Catalysed By The Branched Chain 2-OxoAcid Dehydrogenase Complex

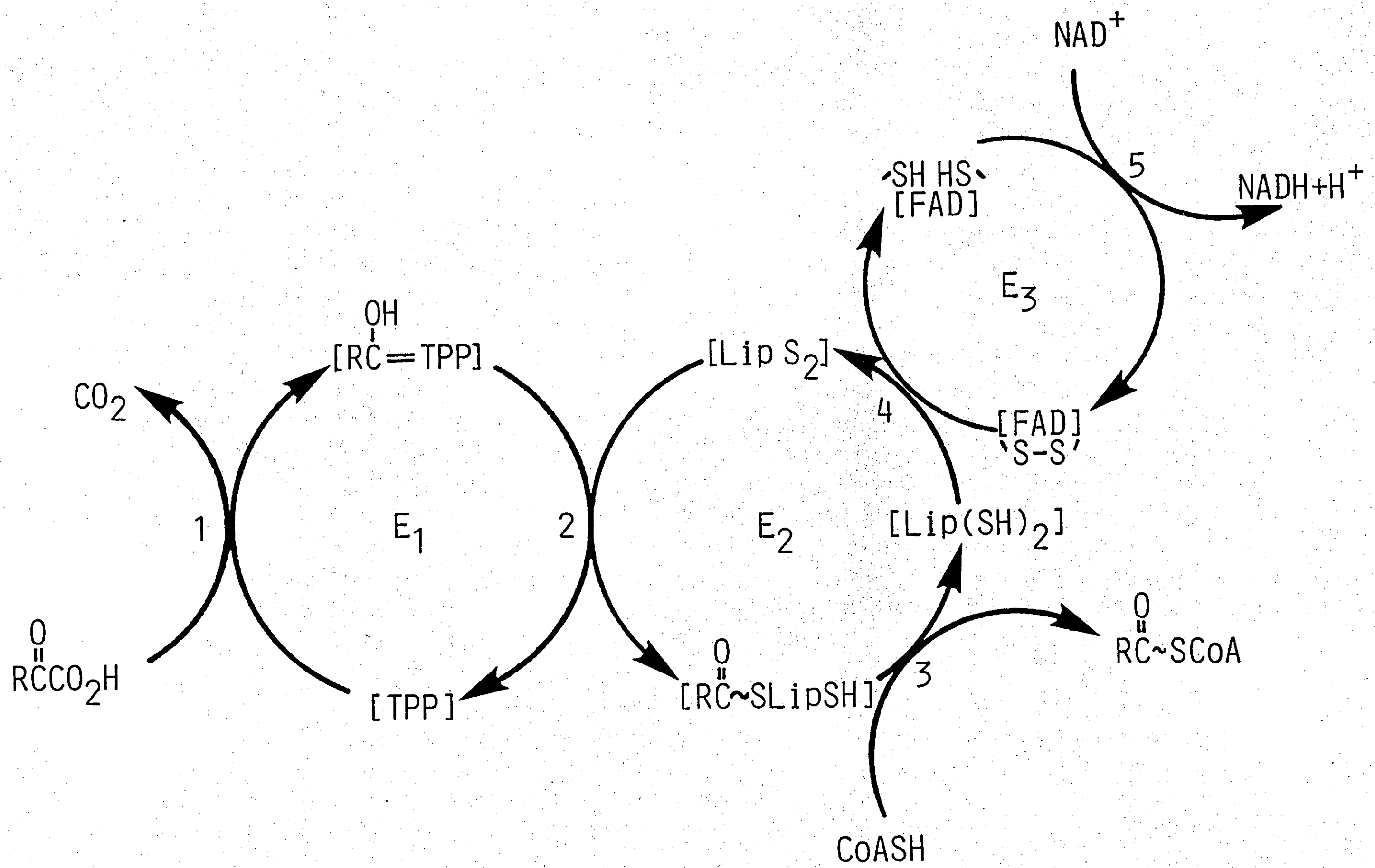
(after Reed and Yeaman, 1987).

TPP, thiamin diphosphate; $LipS_2$ and $Lip(SH)_2$, lipoyl moiety and its reduced form; CoASH, coenzyme A; FAD, flavin adenine nucleotide; NAD^+ and NADH, nicotinamide adenine dinucleotide and its reduced form; E1, branched chain 2-oxoacid decarboxylase; E2, dihydrolipoamide acyltransferase; E3, dihydrolipoamide dehydrogenase.

R = $(CH_3)_2.CH-$; 2-oxoisovalerate (3-methyl-2-oxobutanoate),

R = $(CH_3.CH_2).(CH_3).CH-$; 2-oxo-3-methylvalerate (3-methyl-2-oxopentanoate),

R = $(CH_3)_2.CH.CH_2-$; 2-oxoisocaproate (4-methyl-2-oxopentanoate).



NAD+
SH HS
[FAD]
5
NADH+H+
E3
OH
[RC=TPP]
[Lip S2]
4
[FAD]
S-S
CO2
1
E1
2
E2
[Lip(SH)2]
O
RCCO2H
[TPP]
O
[RC~SLipSH]
3
O
RC~SCoA
CoASH

smaller fragment is derived. The larger of the two fragments exhibits transacylase activity; however proteolytic conversion of this species to the 22000-M_r limit polypeptide renders the inner core catalytically inactive. Thus, the E2 component of the BCOAD complex is apparently analogous to the equivalent components of the pyruvate and 2-oxoglutarate dehydrogenase complexes, whose structures have been investigated more extensively. Limited proteolysis and electron microscopy of the PDH E2 component has revealed that it consists of a compact domain and a flexible extended domain (Bleile *et al*., 1979; Hale and Perham, 1979; Kresze and Ronft, 1980; Bleile *et al*., 1981). The compact domain contains the acetyltransferase active site, and forms the basis of the E2 "inner core" which is visible in the electron microscope. The extended domain, which is readily released from the inner core by limited proteolysis, contains the covalently bound lipoic acid moiety or moieties. Proton nuclear magnetic resonance spectroscopy has provided evidence that the lipoyl and active site domains are connected via a highly mobile segment of the polypeptide (Perham *et al*., 1981; Packman *et al*., 1984). This segment is thought to facilitate interaction of the lipoyl moiety with the active sites of the three component enzymes (Hackert *et al*., 1983).

1.6 REGULATION OF THE BCOAD COMPLEX

The overall activity of the BCOAD complex is regulated by end-product inhibition (by NADH and the appropriate Acyl CoA products), and, in common with the pyruvate dehydrogenase complex, by covalent modification via a phosphorylation/dephosphorylation cycle (Reed *et al*., 1985). BCOAD complex is phosphorylated by a specific kinase which

copurifies with the complex and is tightly bound to the E2 core (Cook *et al*., 1985), although the subunit structure of this regulatory enzyme has not yet been elucidated.

In the presence of Mg^{2+} ions and ATP, the kinase phosphorylates two serine residues which are closely grouped on the α subunit of the E1 component (Cook *et al*., 1984). The V_{max} of the phosphorylated decarboxylase is drastically reduced, resulting in inactivation of the complex. Phosphorylation of one of the two serine residues correlates closely with inactivation; the role of the second phosphorylatable site remains unclear (Cook *et al*., 1983). Recently, the amino acid sequences of the tryptic phosphopeptides containing the inactivation sites of the pyruvate and branched chain 2-oxoacid dehydrogenases have been shown to share considerable homology (Cook *et al*., 1984); however, the phosphopeptide from PDH contains a second phosphorylatable serine residue. This presumably explains why only two sites of phosphorylation are present within the BCOAD complex, compared to the three sites observed in PDH (Yeaman *et al*., 1978).

Dephosphorylation and concomitant reactivation of the BCOAD complex is catalysed by a distinct phosphatase which is relatively loosely attached to its substrate, being lost as a soluble entity during purification of the complex. However, this phosphatase has been purified approximately 8000-fold from extracts of bovine kidney mitochondria (Damuni *et al*., 1984). The highly purified phosphatase has a native M_r of about 460000 as estimated by gel permeation chromatography, but its subunit structure is unknown. In contrast to the PDH phosphatase, the BCOAD complex phosphatase is apparently metal ion-independent. Activity of the latter phosphatase is inhibited by nucleoside tri- and diphosphates, and is stimulated by basic

polypeptides such as protamine, poly (L-lysine) and poly (L-arginine). Damuni and coworkers (Damuni _et al_., 1985; Damuni _et al_., 1986) have recently identified and purified to near homogeneity a 36000-M_r protein inhibitor of the BCOAD complex phosphatase, which could conceivably be of major regulatory significance.

Despite the realisation that the BCOAD complex is regulated by a phosphorylation-dephosphorylation cycle, much remains to be learned concerning the factors which govern the "activity state" or degree of phosphorylation of the complex. The significance of the activity state in determining the level of BCOAD complex activity in a particular tissue is reflected by the finding that, in fed rats, about 70% of the whole-body BCOAD complex activity is located in the liver, although the heart and muscle contain significant amounts of phosphorylated and hence inactive complex (Wagenmakers _et al_., 1984; Patston _et al_., 1986). Furthermore, when rats are fed a low protein diet, a small decrease in the total amount of liver enzyme is detectable, but a substantial decrease in the active form of liver complex is observed, indicating that the decrease in flux through the complex results from increased phosphorylation (Patston _et al_., 1986; Harris _et al_., 1986). It is not known at this stage whether the increased phosphorylation is mediated by changes in the levels of the liver kinase and/or phosphatase, or by allosteric or covalent control of one or both of these regulatory enzymes. In addition, the BCOAD complex "activator protein", described by Fatania _et al_. (1982) and identified as the dissociated, non-phosphorylated E1 component of the complex by Yeaman _et al_. (1984) may have a role in buffering covalent modification of the hepatic BCOAD complex.

1.7 BIOSYNTHESIS OF THE BCOAD COMPLEX

Biosynthesis of the BCOAD complex remains largely uninvestigated, although the E3 component (which is common to all three 2-oxoacid dehydrogenases) has been shown to be synthesised as a higher M_r precursor (Matuda *et al*., 1983; Hunter, 1985).

With the identification of all but two of the protein-coding reading frames in mammalian mitochondrial DNA, and the observation that defects in BCOAD complex activity are inherited as an autosomal recessive disorder, it can be concluded that the four component polypeptides of the BCOAD complex are coded for by nuclear genes. Consequently, the biogenesis of the BCOAD complex is likely to proceed via the general pathway for cytoplasmically-synthesised mitochondrial polypeptides outlined in section 1.2.1.

Key features of the BCOAD complex include the covalently-bound lipoic acid moiety of the E2 component, the presence of TPP and FAD which are non-covalently bound to the E1 and E3 components, respectively, and the highly structured, multimeric array of the native complex. These features are pertinent in the context of BCOAD complex biogenesis, since they raise intriguing questions such as the following; At what stages are the cofactors attached or bound to the appropriate polypeptides? How is complex assembly in the correct submitochondrial location mediated? How is the synthesis and import of the four component polypeptides coordinated? Thus, a detailed knowledge of the biosynthesis of the BCOAD complex would provide information concerning aspects of mitochondrial biogenesis which are at present relatively poorly understood.

1.8 SUCCINATE DEHYDROGENASE

Succinate dehydrogenase (SDH; E.C. 1.3.99.1) occupies a unique position in energy-yielding metabolism, being a component of both the tricarboxylic acid cycle and the electron transport chain. Succinate dehydrogenases have been purified from several mitochondrial and bacterial sources and they invariably comprise two subunits, each containing iron-sulphur centres; a large flavoprotein subunit (M_r 65-72000) containing covalently-bound FAD, and a smaller iron-sulphur protein subunit (M_r 25-30000). Additionally, these subunits may be associated with two small polypeptides (M_r 13500-17000 and 7000-14000) or a cytochrome b (reviewed by Hederstedt and Rutberg, 1981).

The most extensively studied eukaryotic succinate dehydrogenase is probably that from bovine heart mitochondria. A water soluble form of the bovine heart enzyme was isolated by Davis and Hatefi (1971), which consisted of two subunits with M_r values of 70000 and 27000. The large subunit contains one molecule of FAD, which is covalently bound via the 8α-methyl group of the isoalloxazine ring and the N(3) atom of a histidyl residue on the polypeptide chain (Salach *et al*., 1972; Singer and Edmondson, 1974). The active site is also thought to be located on this subunit (Kenney *et al*., 1976).

Biophysical methods have revealed that the bovine heart SDH holoenzyme contains three different Fe-S clusters, which are of the [2Fe-2S], [3Fe-xS] and [4Fe-4S] types. However, definitive evidence concerning their subunit location has yet to emerge (reviewed by Singer and Johnson, 1985).

Studies on succinate dehydrogenase have been performed using

three main types of preparation, namely 1) inner membrane preparations 2) membraneous preparations exhibiting succinate-ubiquinone reductase activity (complex II), and 3) preparations of homogeneous soluble enzyme. In its membrane environment, the enzyme is stabilised against oxygen (Beinert et al., 1977; Ohnishi et al., 1976), shows high succinate-ubiquinone reductase activity which is inhibited by carboxin and thenoyltrifluoroacetone (Ziegler, 1961; Mowery et al., 1977) and has a high turnover number when phenazine methosulphate is used as electron acceptor. The properties of SDH in preparations of complex II and of soluble enzyme differ from each other and from the membrane-bound form of the enzyme.

Preparations of complex II are also stable to oxygen and exhibit ubiquinone reductase activity, but the turnover number of this form of the dehydrogenase in the phenazine methosulphate assay is much lower than that of the enzyme in the inner membrane. Complex II preparations contain, in addition to succinate dehydrogenase, at least two other polypeptides, termed CII-3 and CII-4, which have M_r values of 13500 and 7000 respectively (Capaldi et al., 1977). The oxygen stability and reactivity with ubiquinone is dependent on the presence of these polypeptides (Ackrell et al., 1980).

The soluble enzyme which may be extracted from complex II has an intermediate turnover number using phenazine methosulphate as electron acceptor (Ackrell et al., 1977; Baginsky and Hatefi, 1969), is devoid of ubiquinone reductase activity, and is very labile, so that exposure to oxygen results in destruction of Fe-S centre 3 (Beinert et al., 1977). These changes are coupled with the appearance of a new catalytic activity, a "low K_m" succinate-ferricyanide reductase (Vinogradov et al., 1975) which is functional at low concentrations of

ferricyanide. The latency of this activity is presumed to reflect the inaccessibility of the reaction site when the enzyme is associated with other membrane components.

SDH is tightly associated with the mitochondrial inner membrane and is partially buried within the lipid bilayer. Ferricyanide binding, immunological and chemical labelling studies have indicated that SDH is located both functionally and physically on the matrix side of the inner membrane (Klingenberg and Buchholz, 1970; Merli *et al.*, 1979; Girdlestone *et al.*, 1981). The topographical aspects of succinate dehydrogenase are discussed more fully in Chapter Four.

Despite the considerable attention which has been directed at understanding the structure and enzymology of succinate dehydrogenase, the question of its biosynthesis has scarcely been addressed. When I embarked on this project in October, 1983, only one study concerning the biosynthesis of SDH had been reported (Hattori *et el.*, 1983). This study documented that the SDH large subunit in sweet potato (*Ipomoea batatas*) is synthesised *in vitro* as a precursor with an M_r value which is 3000 greater than the mature form, when sweet potato poly(A)$^+$ RNA is used to direct protein synthesis in a wheat germ cell-free system. Furthermore, the biosynthetic machinery involved in the covalent attachment of FAD to the large subunit and insertion of the Fe-S clusters has yet to be characterised, and the points in the SDH biogenetic pathway at which these prosthetic group attachments occur are completely unknown. Thus, succinate dehydrogenase represents an attractive candidate for research into mitochondrial biogenesis.

1.9 AIMS OF THIS STUDY

Previous sections of this chapter have served to illustrate how few data exist concerning the biogenesis of the branched chain 2-oxoacid dehydrogenase (BCOAD) complex and of succinate dehydrogenase, in comparison with other mitochondrial enzymes. The lack of knowledge is more pronounced when the relatively sophisticated biogenetic pathways of these two dehydrogenases is taken into account. This study was therefore directed at investigating the biosynthesis of the components of the BCOAD complex in cultured mammalian cells, and to perform an analogous study on succinate dehydrogenase.

The question of the topographical arrangement of succinate dehydrogenase within the mitochondrial inner membrane was also addressed, since the availability of specific antisera allowed application of a membrane digestion/immune blotting approach to this problem.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS AND BIOCHEMICALS

Benzamidine-HCl, p-aminobenzamidine-HCl, PMSF, leupeptin, DOC (sodium salt), Tween 20, DNP, TEMED, Coomassie Brilliant Blue R250, branched chain 2-oxoacids (sodium salts), NAD^+, TPP, NEM and antifoam A concentrate were purchased from Sigma Chemical Co., Fancy Rd., Poole, Dorset, U.K. PPO, toluene and sucrose came from Koch-Light Laboratories Ltd., Colnbrook, Berks., U.K.

Tris, CoA (trilithium salt) and DTT were the products of Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Acrylamide, N,N'-methylenebisacrylamide, SDS, Folin Ciocalteu's Phenol reagent and L-cysteine-HCl were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

PEG 6000 was purchased from Serva, Heidelberg, West Germany. Iodogen™ came from Pierce and Warriner (U.K.) Ltd., Chester, U.K. Triton X-100 was purchased from Rohm and Haas (U.K.) Ltd., Croydon, Surrey, U.K. Pyronin Y was obtained from George T. Gurr Ltd., London SW6, U.K. 2-mercaptoethanol was supplied by Riedel-de-Haën, via A. & J. Beveridge Ltd., Edinburgh, U.K. Salicylic acid came from Aldrich Chemical Co., Gillingham, Dorset, U.K. FCCP was provided by Dr. P.G. Heytler, Du Pont De Nemours and Co. (Inc.), Delaware, U.S.A. All other reagents were of the highest grades available commercially.

2.1.2 RADIOCHEMICALS

N-ethyl [2,3-^{14}C]maleimide (10.1mCi/mmol), Na^{125}I (carrier free) and L-[^{35}S]methionine (>1100 Ci/mmol) were obtained from Amersham

International plc, PO Box 16, Amersham, Bucks., U.K.

2.1.3 PROTEINS AND ENZYMES

The following protein and enzyme preparations were obtained from Sigma Chemical Co., Fancy Rd., Poole, Dorset, U.K.:-
bovine serum albumin (essentially fatty acid and globulin free); lipoamide dehydrogenase, type III (E.C. 1.6.4.3) from porcine heart; papain, type III (E.C. 3.4.22.2) from *Papaya latex*; protein A from *Staphylococcus aureus*, Cowan I strain; TLCK-treated α-chymotrypsin, type VII (E.C. 3.4.21.4) from bovine pancreas; TPCK-treated trypsin, type XIII (E.C. 3.4.21.4) from bovine pancreas.

Protease K (E.C. 3.4.21.14) from *Tritirachium album* and lipoamide dehydrogenase (E.C. 1.6.4.3) from porcine heart were from Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Electrophoresis calibration kit for low M_r proteins ("low M_r markers") came from Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K.

Purified bovine heart succinate dehydrogenase was a generous gift from Professor Tsoo E. King, Department of Chemistry, State University of New York at Albany, Albany, NY 12222, U.S.A.

Purified E1 subcomplex from bovine kidney branched chain 2-oxoacid dehydrogenase (BCOAD) complex and some of the native BCOAD complex used in this study were kindly provided by Dr. Steve J. Yeaman, Department of Biochemistry, University of Newcastle Upon Tyne.

2.1.4 MATERIALS FOR CHROMATOGRAPHY

Bio-Gel HTP was purchased from BioRad Laboratories Ltd., Holywell Industrial Estate, Watford, Herts., U.K.

Sephadex G-25 (medium grade) was the product of Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K.

2.1.5 CELL CULTURE MATERIALS

Glasgow-modified Eagle's medium without L-glutamine or without L-glutamine and L-methionine, newborn calf serum, MEM non-essential amino acids (100x), trypsin solution [(10x; 2.5% (w/v)], $NaHCO_3$ solution [(7.5% (w/v)] and Nunc tissue culture flasks and petri dishes were obtained from Gibco Europe Ltd., Renfrew Rd., Paisley, Renfrewshire, U.K.

Sterile glassware and filter-sterilised versene solution (8mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.5, 0.137M NaCl, 2.7mM KCl, 0.5 mM EDTA, 0.0015% (w/v) phenol red) were supplied by the staff of the Wellcome Tissue Culture Unit associated with this department. Trypsin/versene solution consisted of a 10-fold dilution of 10x trypsin solution in versene solution.

2.1.6 PHOTOGRAPHIC MATERIALS

X-Omat S and XAR-5 X-ray films, Kodak FX-40 liquid fixer and Kodak LX-24 X-ray developer were purchased from Kodak Ltd., Dallimore Rd., Manchester, U.K.

Cronex "Lightning-Plus" intensifying screens were purchased from

Du Pont (U.K.) Ltd., Stevenage, Herts., U.K.

Plast-X cassettes, for exposure of X-ray films, were obtained from Anthony Monk (England) Ltd., Sutton-in-Ashfield, U.K.

2.1.7 BIOLOGICAL MATERIALS

PK-15 (pig kidney), NBL-1 (bovine kidney) and BRL (Buffalo rat liver) cell lines were purchased from Flow Laboratories Ltd., Second Avenue Industrial Estate, Irvine, Ayrshire, U.K.

New Zealand White rabbits were provided by the Departmental Animal House. Bovine hearts and kidneys were obtained from Glasgow Abattoir, Duke St., Glasgow, or from Paisley Abattoir, Sandyford Rd., Paisley, Renfrewshire, U.K. Organs were obtained within one hour of the animal's slaughter, transported to the laboratory on ice, and used within two hours of slaughter.

Normal rabbit serum was provided by the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, U.K.

Heat inactivated horse serum was purchased from Gibco Europe Ltd., Renfrew Rd., Paisley, Renfrewshire, U.K.

Pansorbin (standardised 10% (w/v) formalinised *S. aureus* cells) was obtained from Calbiochem-Behring Corp., Bishops Stortford, Herts., U.K.

2.1.8 MISCELLANEOUS

Freund's adjuvants (complete and incomplete) were from Difco Laboratories, Central Avenue, West Molesey, Surrey, U.K.

Nitrocellulose paper (0.45μm pore size) was supplied by

Schleicher and Schüll, via Anderman and Co. Ltd., Laboratory Supplies Division, Kingston-upon-Thames, Surrey, U.K.

Nalgene filter sterilisation units (0.20μm pore size) were from Nalge Company, Rochester, New York, U.S.A.

2.2 METHODS

2.2.1 ASSAY OF BCOAD COMPLEX ACTIVITY

The initial rate of overall BCOAD complex activity was determined by monitoring NADH formation at 340nm and 30°C, as described by Pettit *et al*. (1978). The assay mixture comprised 50mM potassium phosphate buffer, pH 8.0, 2.5mM NAD^+, 0.2mM TPP, 1mM $MgCl_2$, 0.13mM CoASH, 2.6mM cysteine-HCl, 2mM 2-oxoacid (sodium salt) supplemented with 10 units of lipoamide dehydrogenase (Sigma) per ml. The final pH was 7.4. The reaction was initiated by addition of enzyme sample to the assay mixture.

2.2.2 PROTEIN DETERMINATION

Protein was determined using a modification of the procedure of Lowry *et al*. (1951) as described by Markwell *et al*. (1978). Bovine serum albumin was used as a protein standard.

2.2.3 SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

a) Preparation Of Analytical And Preparative Gels

Proteins were electrophoresed on SDS-containing polyacrylamide gels using the discontinuous Tris-glycine buffer system of Laemmli (1970).

For analytical purposes, gels were cast using home-made apparatus in slabs of 19.0cm x 9.5cm x 0.15cm, or when greater resolution was required, using a BioRad Protean™ 16cm apparatus in slabs of 16.0cm x 14.0cm x 0.15cm.

Preparative gels were cast using the home-made apparatus with a thickness of 0.3cm. The stacking gel was cast with a single well of 16.0cm x 1.2cm x 0.3cm.

Resolving gels were polymerised from a solution containing 0.375M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, and acrylamide at a concentration of between 6% (w/v) and 15% (w/v). The ratio of acrylamide to N,N'-methylenebisacrylamide was 36.5:1. The solution was degassed before polymerisation was initiated by adding TEMED to 0.025% (v/v) final concentration. Stacking gels were polymerised from a solution containing 0.12M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.19% (w/v) ammonium persulphate, 0.03% (v/v) TEMED, 5.26% (w/v) acrylamide and 0.14% (w/v) N,N'-methylenebisacrylamide.

b) <u>Preparation Of Samples For Electrophoresis</u>

Protein solutions were mixed with an equal volume of Laemmli sample buffer (0.0625M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 0.001% (w/v) Pyronin Y) containing 5% (v/v) 2-mercaptoethanol or 10mM DTT and boiled for 5 min prior to electrophoresis. When protein samples were in pellet form, these were dissolved in an appropriate volume of Laemmli sample buffer and boiled as above.

c) Conditions Of Electrophoresis

Electrophoresis was performed in a water-cooled apparatus at a constant current of 40-60mA per gel until the Pyronin Y tracker was within 0.5cm of the bottom of the gel. The electrode buffer contained 0.025M Tris-HCl, pH 8.3, 0.192M glycine, 0.1% (w/v) SDS.

d) Staining And Scanning Of Gels

Gels were stained for protein in either 0.1% (v/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid for 1h or 0.04% (w/v) Coomassie Brilliant Blue R250, 25% (v/v) propan-2-ol, 10% (v/v) acetic acid for 16-20h and destained in 20% (v/v) methanol, 10% (v/v) acetic acid.

Densitometric scanning of gels was performed using an LKB 2202 Ultroscan laser densitometer.

2.2.4 RADIOLABELLING TECHNIQUES

a) Modification Of BCOAD Complex With N-ethyl [2,3-^{14}C]maleimide

A portion (200μg) of purified BCOAD complex was diluted to 100μl with 20mM Tris-HCl, pH 7.2 and precipitated overnight with 4 volumes of acetone at -20°C. The precipitate was collected by centrifugation at 14000xg for 2 min. The pellet was air dried, dissolved in 100μl of 20mM Tris-HCl, pH 7.2, 2% (w/v) SDS and boiled for 5 min.

2uCi (0.2μmol) of *N*-ethyl [2,3-^{14}C]maleimide was then added and the reaction was allowed to proceed for 18h at room temperature. The

reaction was terminated by the addition of 2-mercaptoethanol to 5% (v/v) final concentration. Radiolabelled protein was precipitated and air dried as above, and redissolved in 100μl of Laemmli sample buffer (section 2.2.3b) containing 5% (v/v) 2-mercaptoethanol. After boiling for 5 min, a 3μl aliquot was counted for radioactivity (section 2.2.5b) and the remainder was stored at -20°C until required.

b) Iodination Of Protein A And Low Mr Markers

Protein A and low M_r markers were iodinated using the Iodogen™ method of Salacinski *et al*. (1981).

1mg of Iodogen™ was dissolved in 0.5ml of chloroform and coated over the bottom of a glass vial by blowing off the solvent using an air stream. Protein A (1mg) or low M_r markers (578μg) dissolved in 0.5ml of 20mM Tris-HCl, pH 7.2, 0.15M NaCl was added to the Iodogen™-coated vial. The reaction was initiated by adding 300-500μCi of $Na^{125}I$. After 20 min at room temperature, the reaction mixture was gel-filtered on a column of Sephadex G-25 (5.1cm x 1.5cm) equilibrated in 20mM Tris-HCl, pH 7.2, 0.15M NaCl. Fractions containing ^{125}I-labelled protein A were pooled, divided into small aliquots, and stored at -20°C until required.

2.2.5 DETECTION OF RADIOACTIVITY

a) Measurement Of Gamma Radiation

Aqueous samples containing ^{125}I-labelled proteins were counted in an LKB Wallac 1275 minigamma counter.

b) Liquid Scintillation Spectrometry

Aqueous radioactive samples were made up to 0.5ml with distilled water. After the addition of 4.5ml of scintillation fluid (0.5% (w/v) PPO in 35% (v/v) Triton X-100, 65% (v/v) toluene), the samples were counted in a Beckman LS 6800 liquid scintillation counter.

c) Fluorography

Polyacrylamide gels were processed for fluorography using the method of Chamberlain (1979). After fixing for 1h in 50% methanol, 10% (v/v) acetic acid or overnight in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid, the gel was washed over a period of 30 min in five changes of distilled water. The gel was then soaked in 1M sodium salicylate, pH 6.0, for 30 min before being dried down under vacuum onto Whatman 3MM filter paper. The dried gel was exposed to XAR-5 or X-Omat S X-ray film at -80°C in the presence of a Cronex "Lightning-Plus" intensifying screen. Films were developed after exposure for a suitable number of days.

d) Autoradiography

^{125}I-labelled proteins bound to nitrocellulose paper were visualised by exposing the dried paper to X-Omat S X-ray film, as described above.

2.2.6 CELL CULTURE

a) Growth Media

Normal growth medium (NGM) for routine growth of PK-15 cells consisted of Glasgow-modified Eagle's medium supplemented with 5% (v/v) newborn calf serum, 1% (v/v) non-essential amino acids and 0.2% (w/v) $NaHCO_3$. NBL-1 and BRL cells were grown in the same medium, except that the concentration of newborn calf serum was 10% (v/v).

Minus methionine medium (MMM) consisted of Glasgow-modified Eagle's medium (without L-glutamine or L-methionine) supplemented with 1% (v/v) non-essential amino acids, 2mM L-glutamine, and newborn calf serum at the appropriate concentration for each cell line, as described above. Low methionine medium (LMM) consisted of 5% (v/v) normal growth medium, 95% minus methionine medium.

b) Routine Maintenance Of Cells

Cells were routinely grown in NGM in $175cm^2$ plastic tissue culture (Roux) flasks at $37^{\circ}C$ in an atmosphere of 95% air/5% CO_2 and were subcultured when confluent monolayers were observed (generally every 3-5 days).

Cell monolayers were rinsed once with versene solution (section 2.1.5), once with 5ml of trypsin/versene solution (section 2.1.5) then incubated with approx. 0.5ml of trypsin/versene solution until complete detachment of the cells from the flask surface was observed. 10ml of NGM was then added to the flask and the cells were gently passaged up and down a sterile pipette until a suspension of single

cells was obtained. A sample of the cell suspension was counted using a haemocytometer and 3-5 x 10^6 cells were returned to the flask in approx. 50ml NGM.

c) Metabolic Labelling Of Cells With [^{35}S]Methionine

Sterile plastic petri dishes (10cm diam.) were seeded with 10ml of NGM containing cells at a concentration of 3 x 10^5 cells/ml. The petri dishes were incubated at 37°C in an atmosphere of 95% (v/v) air/5% (v/v) CO_2 for 24-48h until the monolayers were semi-confluent. The monolayers were rinsed once with 4ml of LMM, then incubated for 90 min at 37°C with a further 4ml of LMM. After addition of L-[^{35}S]methionine (100-200μCi), dishes were incubated at 37°C for either 4h or overnight. After the appropriate labelling period, cell lysates were prepared as described in section 2.2.7b.

d) Metabolic Labelling Of Cells With [^{35}S]Methionine In The Presence Of Uncouplers Of Oxidative Phosphorylation

Cells were preincubated with LMM as above. 10 minutes prior to the addition of isotope, DNP or FCCP was added to the medium from 100x ethanolic stock solutions to give final concentrations of 1-2mM or 10μM, respectively. After labelling for 4h, cell lysates were prepared as described in section 2.2.7b.

For pulse-chase experiments, monolayers were labelled in the presence of uncouplers as above, rinsed once with 10ml of warm (37°C) NGM, then incubated at 37°C with a further 10ml of NGM with or without uncoupler, as required. After the appropriate chase periods, cell

lysates (section 2.2.7b) were prepared.

2.2.7 PREPARATION OF CELLULAR EXTRACTS AND SUBFRACTIONS

a) Isolation Of Mitochondria From Cultured Cells

The method of Attardi and Ching (1979) was used to obtain crude mitochondrial and post-nuclear supernatant fractions from cultured BRL, NBL-1, or PK-15 cells. All operations were performed at $4^{o}C$.

To prepare mitochondria, the cell monolayers from two 2.5l capacity glass roller bottles were each rinsed twice with 20ml of PBS (20mM potassium phosphate, pH 7.4, 0.15M NaCl) and then scraped off into the same volume of PBS. The cells were pooled and pelleted by centrifugation at 1500xg for 15 min. The cells were then washed twice in 20ml of 0.13M NaCl, 5mM KCl, 1mM $MgCl_2$ before being resuspended in six volumes of 10mM Tris-HCl, pH 6.7, 10mM KCl, 0.15mM $MgCl_2$. After two minutes on ice, the cells were homogenised with three passes of a Potter-Elvenhjem teflon/glass homogeniser. Cell breakage was confirmed by examining the homogenate under a light microscope. After addition of sucrose to 0.25M final concentration, the homogenate was centrifuged at 1100xg for 3 min to remove nuclei. Mitochondria were pelleted from the supernatant fluid by centrifugation at 5000xg for 10 min. The pellet was suspended in 2ml of 10mM Tris-HCl, pH 6.7, 0.15mM $MgCl_2$, 0.25M sucrose. The last two steps were repeated before the final mitochondrial pellet was resuspended in 1ml of 0.0625M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose and a small aliquot was taken for protein determination (section 2.2.2). The protein concentration of the remaining solution was adjusted to 4.0mg/ml and the composition of the buffer was adjusted to that of Laemmli sample

buffer (section 2.2.3b) containing 10mM DTT.

b) Isolation Of Post-Nuclear Supernatant Fractions From Cultured Cells

The cell monolayer from a $175cm^2$ plastic Roux flask was rinsed twice with 10ml of PBS (section 2.2.7a) and then scraped off into 20 ml of PBS. After pelleting the cells by centrifugation at 1500xg for 5 min, they were lysed in 1ml of 0.1M Tris-HCl, pH 8.2, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100 and nuclei were removed by centrifugation at 1100xg for 5 min. An aliquot of the resulting supernatant fraction was utilised for protein determination (section 2.2.2) and the remainder was precipitated overnight with 4 volumes of acetone at -20^oC.

c) Preparation Of [^{35}S]Methionine-Labelled Cell Lysates

The following buffers were filter-sterilised using a nalgene filter (0.2μm pore size):

PBS: 20mM potassium phosphate, pH 7.4, 0.15M NaCl.

Triton-TKM: 0.1M Tris-HCl, pH 8.2, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100.

3D-Lysis: 0.1M Tris-HCl, pH 8.2, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100, 1% (w/v) SDS, 2% (w/v) DOC.

Each buffer was supplemented with 1mM PMSF and 1mM p-amino-benzamidine-HCl immediately before use, and all operations were performed at 4^oC.

Cell monolayers were washed three times with approx. 4ml of PBS. 1ml of Triton-TKM buffer per petri dish was added and the lysed cells were scraped off using a rubber policeman. Nuclei were removed by centrifugation at 1000xg for 5 min. The resulting supernatant fraction was then mixed with an equal volume of 3D-Lysis buffer and centrifuged at 27000xg for 30 min. A 5µl aliquot of the final supernatant was counted for radioactivity (section 2.2.5b) and the remainder was stored at -80°C until required.

d) Preparation Of Bovine Heart Mitochondria

Mitochondria were isolated from bovine heart by the method of Smith (1967). All operations were performed at 4°C. 300g of heart tissue was freed of fat and connective tissue and cut into cubes of approx. 5cm. The cubes of tissue were passed through a mincer and the minced tissue was placed in 400ml of 0.01M Tris-HCl, pH 7.8, 0.25M sucrose (solution A). The pH of the solution was immediately adjusted to 7.5±0.1 by adding 2M Tris base. Excess solution A was removed by placing the minced tissue in a double layer of cheesecloth and squeezing. The sucrose-free material was suspended in 400ml of 0.01M Tris-HCl, pH 7.8, 0.25M sucrose, 1mM Tris-succinate, 0.2mM EDTA (solution B). 3ml of 2M Tris base was added and the suspension was blended in a Philips food blender for 15s. After the addition of a further 3ml of 2M Tris base, the material was reblended for 5s. The blended material was adjusted to pH 7.8 using 2M Tris base, and then centrifuged at 1000xg for 15 min to sediment debris. The resulting supernatant was centrifuged at 5000xg for 30 min to pellet mitochondria. The mitochondria were washed a further once or twice by

resuspending in a small volume of solution B and centrifuging at 5000xg for 15 min. Mitochondria were routinely stored in pellet form at -80°C until required.

For protease digestion experiments, mitochondria were kept at 4°C at a protein concentration of 4.0mg/ml and used within 24h of isolation.

e) Preparation Of Bovine Heart Submitochondrial Particles

Bovine heart mitochondria were thawed from -80°C and resuspended at a protein concentration of approx. 10mg/ml in 50mM sodium phosphate, pH 7.5. All further manipulations were performed at 4°C. The mitochondria were then pelleted by centrifugation at 5000xg for 15 min. The resuspension/centrifugation step was repeated once and the resulting pellet was resuspended in phosphate buffer at approx. 10mg/ml protein concentration.

To generate submitochondrial particles, the mitochondrial suspension was subjected to three 15s bursts of sonication, at 15s intervals, using an MSE sonicator at amplitude setting 3 (high power). The sonicated material was centrifuged at 10000xg for 10 min to pellet unbroken mitochondria. The supernatant fraction was recentrifuged at 100000xg for 1h and the final pellet was resuspended in 50mM sodium phosphate, pH 7.5 at a protein concentration of 4.0mg/ml. Submitochondrial particles were stored in 1.1ml aliquots at -80°C until required.

2.2.8 IMMUNOLOGICAL PROCEDURES

a) Immune Blotting

The immune blotting procedure of Towbin et al. (1979) as modified by Batteiger et al. (1982), was employed to allow immunological detection of polypeptides after their electrophoretic transfer from polyacrylamide gels to nitrocellulose paper.

Proteins were resolved by SDS/polyacrylamide gel electrophoresis (section 2.2.3) and then transferred electrophoretically onto nitrocellulose paper at 400mA for 3h or 40mA for 16-20h using a BioRad Trans-Blot™ cell. The transfer buffer comprised 25mM Tris, pH 8.3, 192mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol, and was cooled to $4^{o}C$ before use.

After electrophoretic transfer, nitrocellulose-bound polypeptides were visualised by staining the paper in 0.1% (w/v) Amido Black, 45% (v/v) methanol, 10% (v/v) acetic acid for 5 min followed by destaining in 45% (v/v) methanol, 10% (v/v) acetic acid for 5 min. Excess binding sites on the paper were then blocked by incubation with 20mM Tris-HCl, pH 7.2, 0.15M NaCl, 0.5% (v/v) Tween 20, 0.005% (w/v) NaN_3 (blocking buffer) for 1h at room temperature or overnight at $4^{o}C$. The nitrocellulose was then incubated with blocking buffer supplemented with 5% (v/v) heat inactivated horse serum and antiserum at a dilution of 1:50-1:100 for 90 min at room temperature. Excess antibody was removed by washing with five changes of blocking buffer over a period of 45 min, before incubation for 90 min at room temperature with blocking buffer containing ^{125}I-labelled protein A (approx. 3×10^6 c.p.m. per blot). Excess ^{125}I-labelled protein A was removed by washing with blocking buffer as above. The processed blot was allowed

to dry at room temperature prior to analysis by autoradiography (section 2.2.5d).

b) <u>Immunoprecipitation From [^{35}S]Methionine-labelled Cell Lysates</u>

Aliquots of [^{35}S]methionine-labelled cell lysates (section 2.2.7c) containing 10^7 c.p.m. (100-600μl) were incubated with 10μl of antiserum for 2h at room temperature. 20μl of Pansorbin (equilibrated in 0.1M Tris-HCl, pH 8.2, 0.1M KCl, 1% (v/v) Triton X-100 , 0.5% (w/v) SDS, 1% (w/v) DOC, 5mM $MgCl_2$ - "3D-TKM buffer") was added and the mixture incubated for a further 1h at room temperature with occasional mixing. Pansorbin was then pelleted by centrifugation at 14000xg for 2 min. Each pellet was resuspended in 1ml of 3D-TKM and recentrifuged as above. The wash in 3D-TKM was repeated twice, and the pellets were finally washed in 1ml of 10mM Tris-HCl, pH 7.4. The final pellets were resuspended in 50μl of Laemmli sample buffer (section 2.2.3b) containing 10mM DTT and boiled for 5 min. A 5μl sample of the supernatant fraction obtained after boiling was counted for radioactivity (section 2.2.5b) and the remainder was analysed by SDS/polyacrylamide gel electrophoresis (section 2.2.3) and fluorography (section 2.2.5c) or stored at -80°C.

CHAPTER THREE

IMMUNOLOGICAL AND BIOSYNTHETIC STUDIES ON SUCCINATE DEHYDROGENASE

3.1 INTRODUCTION

A key tool in the majority of studies on mitochondrial biogenesis has been a high titre, monospecific antiserum to the mitochondrial protein of interest. To be of value, it is desirable that such an antiserum meets the following requirements; a) it should be capable of recognising the precursor form of the antigen to which it was raised, and b) it should exhibit cross-reactivity with equivalent antigens from sources different to that of the antigen. The antiserum is most commonly used to immunoprecipitate radiolabelled antigens which are subsequently analysed by SDS/polyacrylamide gel electrophoresis and fluorography.

When immunological detection of mitochondrial polypeptides is performed *in vitro*, using isolated mRNA to direct protein synthesis in a cell-free system, the primary translation product is the precursor form of the mitochondrial polypeptide. However, *in vivo* studies are hindered by the fact that most precursors have a short half-life, so that they rarely accumulate outside the mitochondrion under normal conditions. As described in Chapter One (section 1.2.5), import of polypeptides which are inserted into or translocated across the mitochondrial inner membrane requires an electrochemical potential gradient across that membrane. Therefore, by employing uncouplers of oxidative phosphorylation to dissipate the transmembrane gradient we can effect accumulation of precursors, provided that these molecules are relatively stable in the cytosolic compartment of the cell. This approach was adopted to investigate the *in vivo* biosynthesis of succinate dehydrogenase.

This chapter describes a) the production of antiserum to native succinate dehydrogenase from bovine heart and its individual large and

small subunits, b) demonstration of the cross-reactivity of these antisera with the subunits of SDH in mammalian cell lines and c) immunoprecipitation of the SDH subunits from BRL and PK-15 cells metabolically labelled with [^{35}S]methionine, and the corresponding precursor forms from cells labelled in the presence of uncouplers of oxidative phosphorylation.

3.2 METHODS

3.2.1 PREPARATION OF ANTISERUM TO SDH AND ITS INDIVIDUAL LARGE AND SMALL SUBUNITS

Antiserum to native succinate dehydrogenase (anti-SDH serum) was prepared as follows. Purified SDH (0.5mg) in 50mM sodium phosphate, pH 7.4 was made up to 0.6ml with 0.9% (w/v) NaCl and mixed with an equal volume of Freund's complete adjuvant. The mixture was injected subcutaneously at multiple sites on the neck and back of a New Zealand White rabbit. 0.5mg portions of enzyme in Freund's incomplete adjuvant were administered at 2-3 week intervals thereafter. Two weeks after the fourth injection, blood was drawn from a marginal ear vein. After standing at 4°C overnight, the serum fraction was centrifuged at 1500xg for 10 min, then stored in 1.0ml aliquots at -20°C until required. Before subsequent bleedings the rabbit was injected two weeks previously with 0.2-0.5mg of enzyme in Freund's incomplete adjuvant.

Subunit-specific antisera to the individual large and small subunits of succinate dehydrogenase (anti-L serum and anti-S serum, respectively) were prepared as follows. Portions of SDH (1.3mg) were made up to 1.0ml with Laemmli sample buffer (section 2.2.3b)

containing 10mM DTT and boiled for 5 min prior to resolution by preparative SDS/polyacrylamide gel electrophoresis (section 2.2.3). The Coomassie blue-stained bands corresponding to the large and small subunits (Fig.3.1) were excised from the gel, soaked in three changes (5 min each) of distilled water, sliced into small pieces and ground to a powder with a small volume of liquid nitrogen using a mortar and pestle. The powder was weighed and stored frozen at $-20^{\circ}C$.

Before injection, an amount of powder corresponding to half of one band was homogenised in 0.75ml of distilled water and mixed with an equal volume of Freund's complete adjuvant. The mixture was injected subcutaneously at multiple sites on the neck and back of the rabbit. This treatment was repeated at 2 week intervals thereafter, with the fourth injection being in Freund's incomplete adjuvant. Blood was collected two weeks after the fourth injection, and serum was obtained as described above. Similar amounts of antigen were administered in Freund's incomplete adjuvant two weeks before subsequent bleedings.

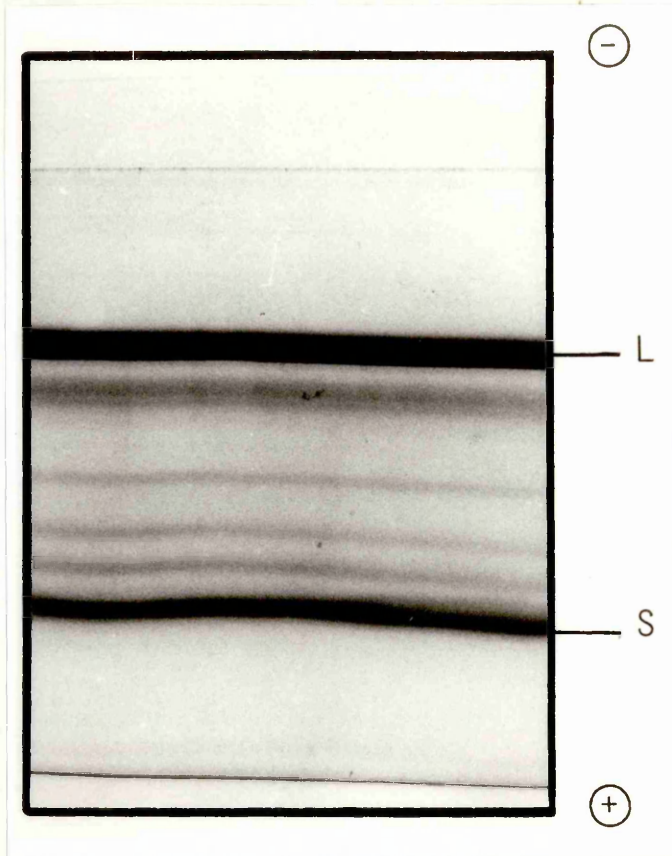
3.3 RESULTS

3.3.1 IMMUNE BLOT ANALYSIS OF PURIFIED SUCCINATE DEHYDROGENASE WITH ANTI-SDH SERUM AND SUBUNIT-SPECIFIC ANTI-L AND ANTI-S SERA

Fig.3.2A shows the Coomassie blue profile of different amounts of the purified succinate dehydrogenase preparation after electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel. In addition to the major M_r 70000 and M_r 27000 bands (corresponding to the large and small subunits of SDH, respectively), the preparation was found to contain several minor contaminants with intermediate M_r values. By the

Fig.3.1 Preparative SDS/Polyacrylamide Gel Electrophoresis Of Bovine Heart Succinate Dehydrogenase

A sample of succinate dehydrogenase (1.3mg) was subjected to electrophoresis on a preparative 10% (w/v) SDS/polyacrylamide gel (section 2.2.3). After staining the gel with Coomassie blue, bands were excised and used for preparation of subunit-specific antisera (section 3.2).


−
L
S
+

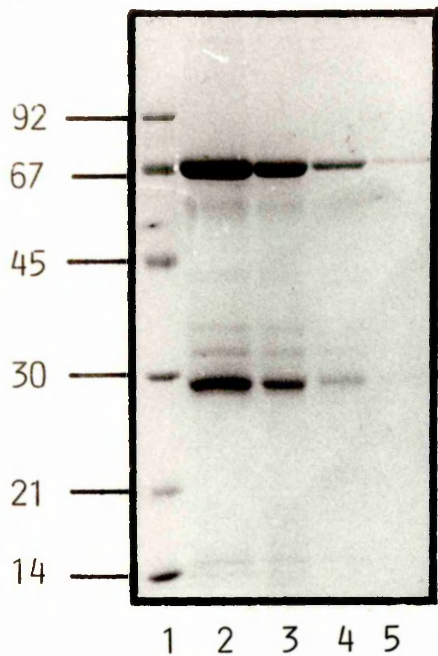
criterion of densitometric scanning the preparation was judged to be 90% pure. Fig.3.2 panels B-D show the results of immune blot analysis of the purified SDH with anti-SDH serum (B) or subunit-specific anti-L (C) or anti-S (D) serum, after detection of immune complexes with ^{125}I-labelled protein A. In addition to reaction with their parent antigens, anti-SDH and anti-L serum both exhibited cross-reaction with all the minor bands in the preparation. Thus, the contaminant polypeptides appear to be immunologically related to the M_r 70000 polypeptide, and are most likely proteolytic fragments of this subunit. Fig.3.2D shows that, at higher loadings of SDH, anti-S serum exhibits weak cross-reactivity with the M_r 70000 subunit and with an M_r 50000 large subunit fragment. These observations are discussed more fully in the last section of this chapter (section 3.4).

3.3.2 IMMUNOLOGICAL DETECTION OF THE SUBUNITS OF SUCCINATE DEHYDROGENASE IN SUBCELLULAR FRACTIONS FROM CULTURED CELLS

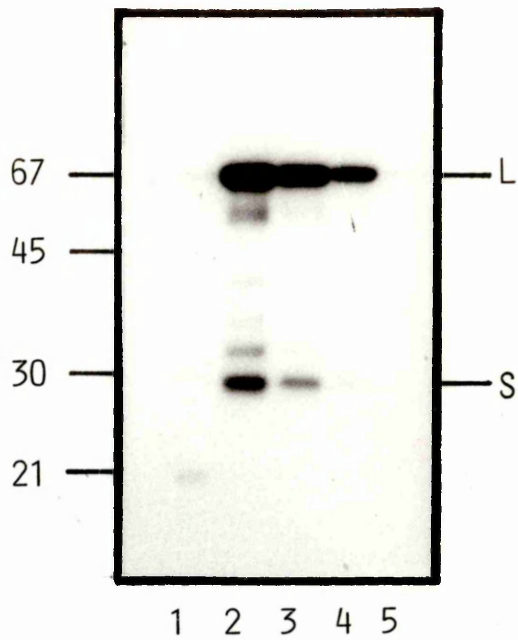
For a successful investigation of the *in vivo* biosynthesis of succinate dehydrogenase in mammalian cell lines, the antisera produced to native bovine heart SDH and its individual subunits would have to show cross-reactivity with the corresponding polypeptides in extracts of the cultured cells. To test whether this was the case, post-nuclear supernatant and mitochondrial fractions from BRL, NBL-1 and PK-15 cell lines were prepared (section 2.2.7) and samples of these fractions were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels (Fig.3.3). Panel A shows the Coomassie blue profile of these samples while panels B-D show the results of immune blot analysis performed with anti-SDH (B), anti-L (C) or anti-S (D) serum.

Fig.3.2 Immuneblot Analysis Of Purified Succinate Dehydrogenase With Anti-SDH Serum And Subunit-Specific Anti-L And Anti-S Sera

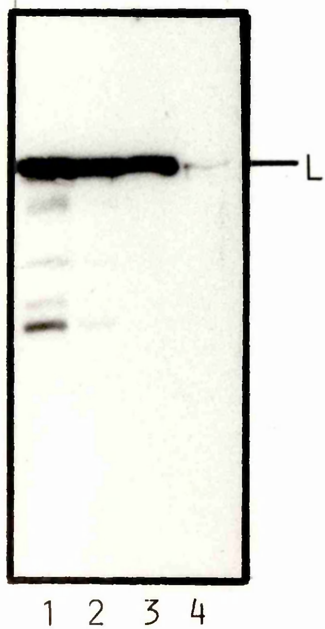
Varying amounts of purified SDH were electrophoresed on a 10% (w/v) SDS/polyacrylamide slab gel (section 2.2.3). One portion of the gel (A) was stained with Coomassie blue. Polypeptides on replicate portions were transferred electrophoretically onto nitrocellulose paper for incubation with anti-SDH (B), anti-L (C) or anti-S (D) serum (section 2.2.8a). Immune complexes were detected by autoradiography (section 2.2.5.d) after incubation with ^{125}I-labelled protein A. Lanes 2-5 (A) 25, 10, 5 or 1μg of enzyme, respectively. Lanes 2-5 (B and D) or 1-4 (C) 5, 2, 1 or 0.2μg of enzyme, respectively. Lane 1, low M_r marker proteins (A) or ^{125}I-labelled low M_r marker proteins (B and D).



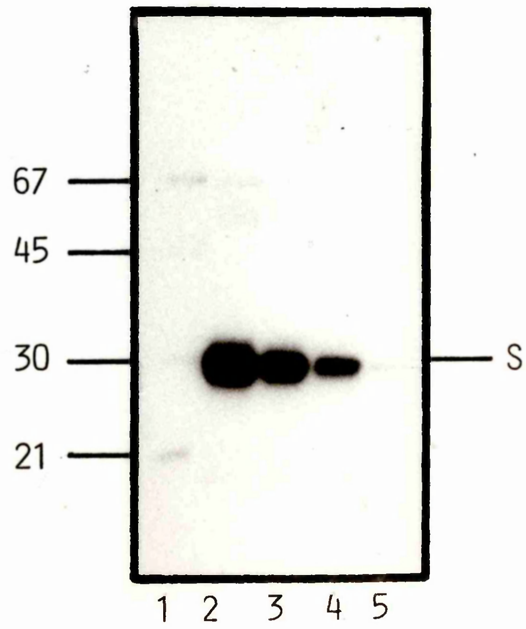
$M_r \times 10^{-3}$
A
92
67
45
30
21
14
L
S
1 2 3 4 5



$M_r \times 10^{-3}$
B
67
45
30
21
L
S
1 2 3 4 5



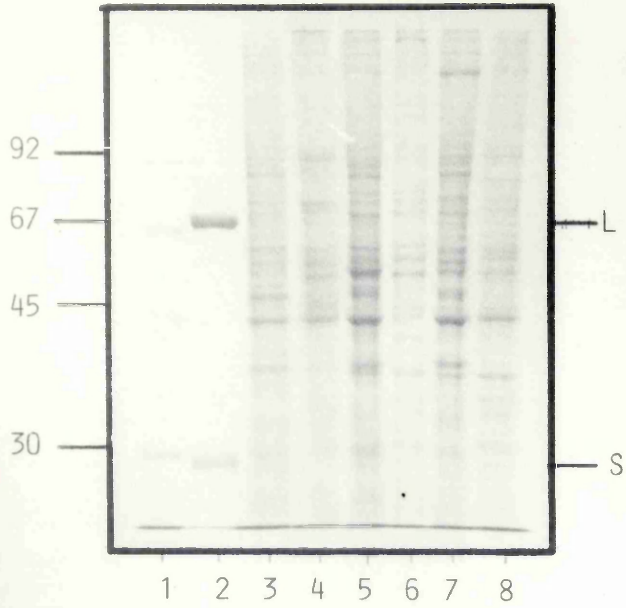
C
L
1 2 3 4



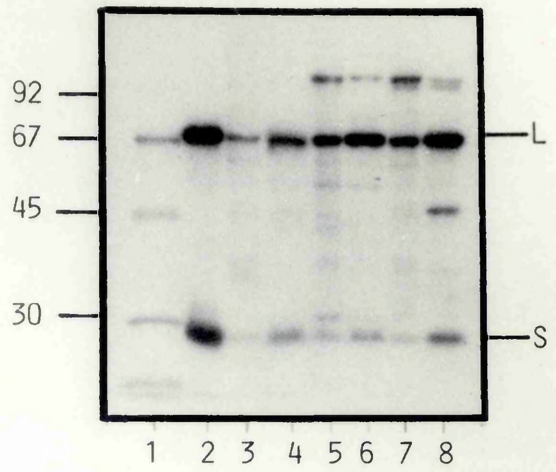
D
67
45
30
21
S
1 2 3 4 5

Fig.3.3 <u>Immunological Detection Of The Large (L) And Small (S) Subunits Of Succinate Dehydrogenase In Cultured Cell Subfractions</u>

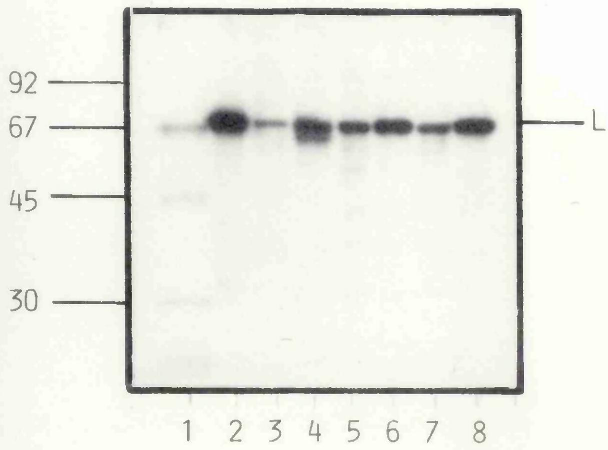
Post-nuclear supernatant and mitochondrial fractions were prepared from cultured BRL, PK-15 and NBL-1 cells (section 2.2.7). Samples of the extracts were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels. A portion of one gel (A) was stained with Coomassie blue. Replicate portions of gel were processed for detection of immunoreactive polypeptides using anti-SDH (B), anti-L (C) or anti-S (D) serum. Lane 1, low M_r markers (A) or ^{125}I-labelled low M_r markers (B-D); lane 2, 10μg (A) or 0.5μg (B-D) purified SDH; lane 3, BRL post-nuclear supernatant fraction (80μg); lane 4, BRL m(80μg)ndria (40μg); lane 5, PK-15 post-nuclear supernatant fraction (80μg); lane 6, PK-15 mitochondria (40μg); lane 7, NBL-1 post-nuclear supernatant fraction (80μg); lane 8, NBL-1 mitochondria (40μg).



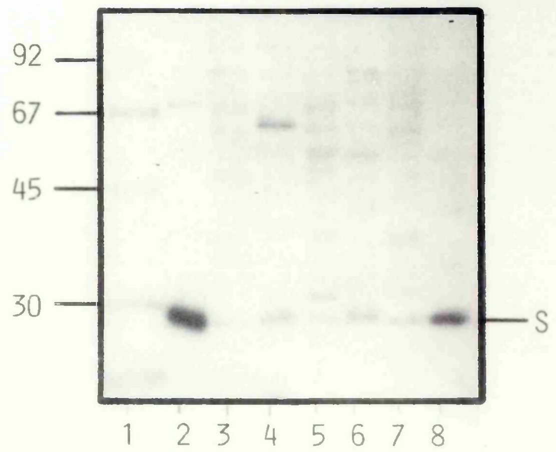
M_r x 10^{-3}
A
92
67
45
30
L
S
1 2 3 4 5 6 7 8



M_r x 10^{-3}
B
92
67
45
30
L
S
1 2 3 4 5 6 7 8



C
92
67
45
30
L
1 2 3 4 5 6 7 8



D
92
67
45
30
S
1 2 3 4 5 6 7 8

These data show that each antiserum permits detection of the corresponding parent antigen(s) in mitochondrial or post-nuclear supernatant fractions from each cell line tested. Additional, weaker bands were observed with each antiserum, particularly with anti-S serum. The strength of the signals from the pig kidney and bovine kidney samples were comparable, in contrast to the relatively weaker signals obtained from the corresponding Buffalo rat liver samples. It was not determined whether differences in signal strength reflected differences in cross-reactivity or different levels of the subunits of SDH in the three cell lines.

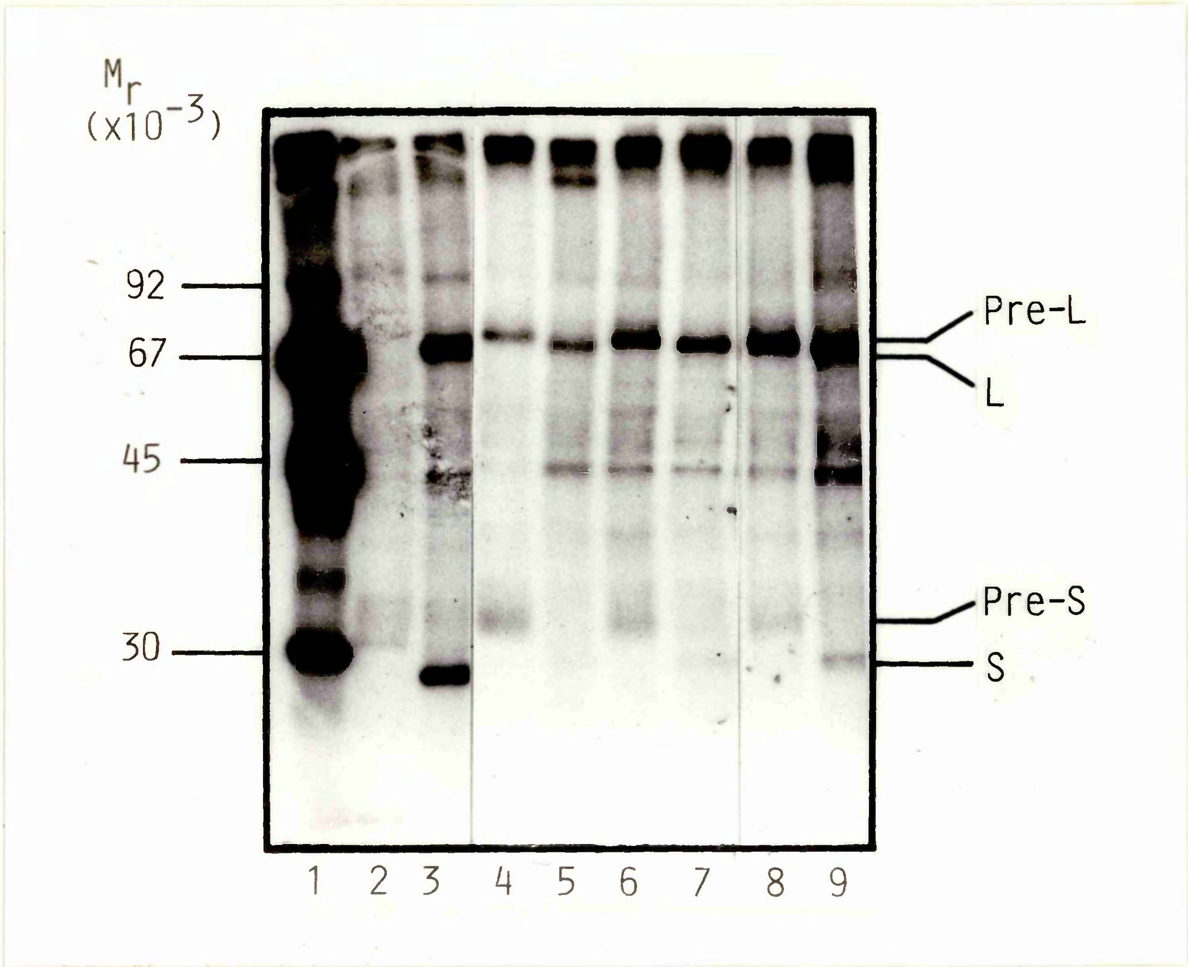
It was also apparent from this analysis was that there was no striking difference in the electrophoretic mobility of either subunit of SDH in the rat (lanes 3 and 4) porcine (lanes 5 and 6) or bovine kidney (lanes 7 and 8) cell lines when compared with the bovine heart enzyme (lane 2).

3.3.3 IMMUNOPRECIPITATION OF NEWLY-SYNTHESISED AND PROCESSED SUBUNITS OF SUCCINATE DEHYDROGENASE IN PULSE-LABELLED AND PULSE-CHASED BRL CELLS

Cultured BRL cells were incubated with [^{35}S]methionine for 3h, then a radiolabelled cell lysate was prepared as described in section 2.2.7c. After treatment of an aliquot of this extract with anti-SDH serum (section 2.2.8b) and analysis of the recovered [^{35}S]methionine-labelled polypeptides by SDS/polyacrylamide gel electrophoresis and fluorography, only two major bands, with M_r values of 70000 and 27000 were observed (Fig.3.4, lane 3). When a second aliquot of extract was treated with non-immune serum, these two bands were absent from the

Fig.3.4 Immunoprecipitation Of Newly-Synthesised And Processed Subunits Of Succinate Dehydrogenase From Pulse-Labelled And Pulse-Chased BRL Cells

BRL cells were pulsed for 3h with [^{35}S]methionine (200µCi/dish) both in the presence and absence of uncoupler, or pulse-labelled in the presence of uncoupler and then chased for 45 min after removal of uncoupler. After preparation of [^{35}S]methionine-labelled cell extracts (section 2.2.7c), indirect immunoprecipitation was performed using non-immune or anti-SDH serum and formalinised *S. aureus* cells (section 2.2.8b). The resulting immunoprecipitates were resolved on a 10% (w/v) SDS/ polyacrylamide gel, and visualised by fluorography (section 2.2.5c). Lane 1, ^{125}I-labelled low M_r markers; lane 2, 3h pulse, non-immune serum; lanes 3-9, immunoprecipitates obtained with anti-SDH serum; lane 3, pulse, no uncoupler; lane 4, pulse + 1mM DNP; lane 5, as 4, but with 45 min chase; lane 6, pulse + 2mM DNP; lane 7, as 6, but with 45 min chase; lane 8, pulse + 10µM FCCP; lane 9, as 8, but with 45 min chase.



M_r
$(x10^{-3})$
92
67
45
30
Pre-L
L
Pre-S
S
1 2 3 4 5 6 7 8 9

resulting immunoprecipitate (lane 2), confirming their identification as the large and small subunits of SDH. When a similar labelling experiment was performed in the presence of 1 or 2mM DNP (lanes 4 and 6, respectively), newly-synthesised (precursor) forms of the large and small subunits, exhibiting M_r values approximately 1-2000 and 4-5000 greater than the corresponding mature forms, were immunoprecipitated. An identical pattern was obtained when the cells were labelled in the presence of 10μM FCCP (lane 8).

To monitor processing of the precursors to the subunits of SDH, BRL cells were labelled with [^{35}S]methionine in the presence of uncouplers and then chased for 45 min in the absence of uncoupler. Lanes 5, 7 and 9 show the products recovered from cells where the chase was preceded by a 3h pulse in the presence of 1mM DNP, 2mM DNP and 10μM FCCP, respectively. In each case, complete conversion of the precursor forms of both subunits to the corresponding mature forms was observed.

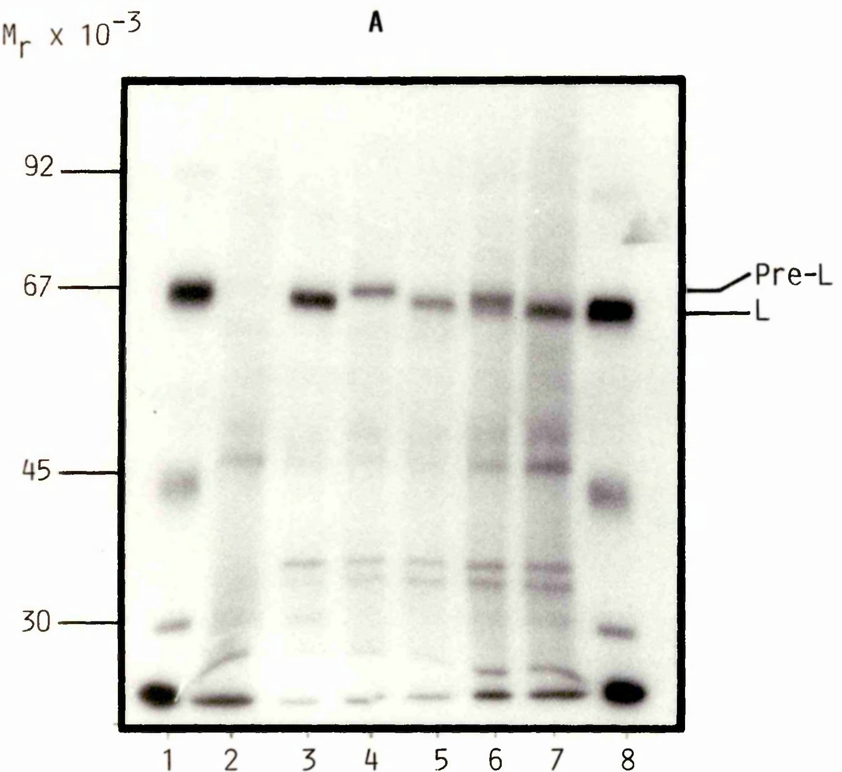
3.3.4 BIOSYNTHETIC STUDIES IN PK-15 CELLS

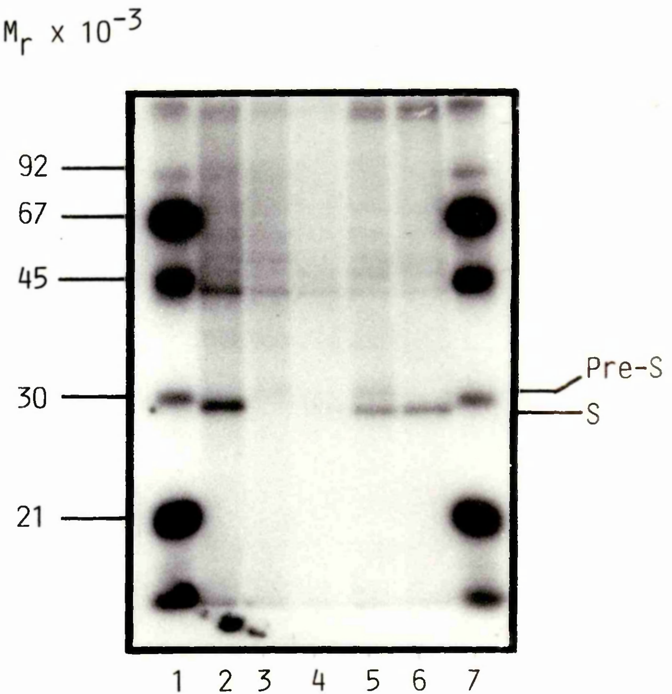
a) Immunoprecipitation Of Newly-Synthesised And Processed Subunits Of SDH In Pulse-Labelled And Pulse-Chased PK-15 Cells

Fig.3.5 demonstrates that higher M_r precursor forms of the large and small subunits of SDH could also be detected in PK-15 cells which had been pulse-labelled with [^{35}S]methionine in the presence of uncouplers. The data shows a) the M_r values of the precursor forms of each subunit in this cell line are similar, if not identical, to the corresponding precursors in BRL cells; b) complete conversion of the precursor forms to the

Fig.3.5 Immunoprecipitation Of Newly-Synthesised And Processed Subunits Of Succinate Dehydrogenase In Pulse-Labelled And Pulse-Chased PK-15 Cells

PK-15 cells were pulse-labelled for 4h with [^{35}S]methionine both in the absence and presence of uncoupler, or pulse-labelled in the presence of uncoupler and then chased for 45 min after uncoupler removal. After preparation of radiolabelled cell lysates, immunoprecipitation was performed using anti-L serum (A) and anti-S serum (B). The resulting immunoprecipitates were analysed by SDS/polyacrylamide electrophoresis on 6% (w/v) (A) or 10% (w/v) (B) slab gels followed by fluorography. Panel A; lanes 1 and 8, ^{125}I-labelled low M_r markers; lane 2, pulse (no uncoupler), non-immune serum; lane 3, pulse, no uncoupler; lane 4, pulse + 2mM DNP; lane 5, as 4, but with 45 min chase; lane 6, pulse + 10μM FCCP; lane 7, as 6, but with 45 min chase. Panel B; lanes 1 and 7, ^{125}I-labelled low M_r markers; lanes 2-6, as lanes 3-7 in A except with anti-S serum.


A
Mr x 10-3
92
67
45
30
Pre-L
L
1 2 3 4 5 6 7 8


B
Mr x 10-3
92
67
45
30
21
Pre-S
S
1 2 3 4 5 6 7

corresponding mature forms occurs during a 45 min chase in the absence of uncoupler; c) pulse-labelling of PK-15 cells in the presence of 10μM FCCP results in only partial accumulation of precursors; d) the quality of the immunoprecipitates obtained from PK-15 cells were superior to those obtained from BRL cells, since fewer contaminating polypeptides were found to co-precipitate with the bona fide antigens. The pig kidney cell line was therefore utilised in further experiments designed to investigate the stability and kinetics of processing of the SDH large subunit precursor.

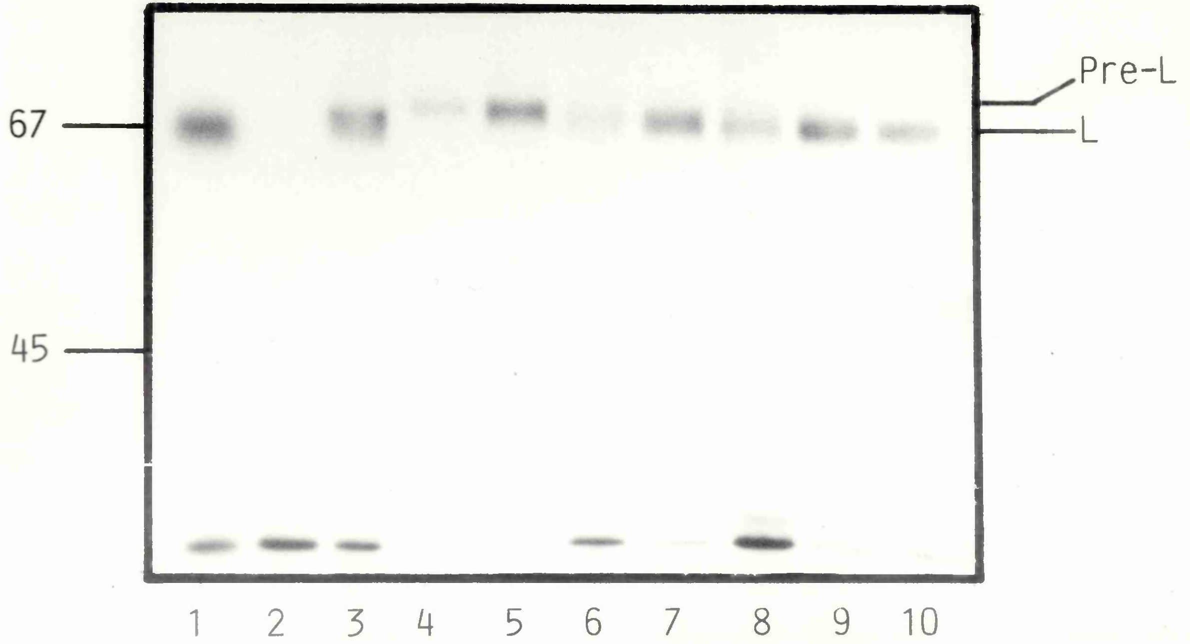
b) Kinetics Of Processing Of The SDH Large Subunit Precursor After Reversal Of Precursor Accumulation

Fig.3.6 shows the result of an experiment in which precursor accumulation in the presence of 2mM DNP was followed by chases of increasing duration in the absence of uncoupler. Lane 4 shows the precursor accumulated at the start of the chase after labelling for 4h in the presence of DNP; lanes 5-10 show the products recovered after 2, 5, 7.5, 10, 15 and 30 min chases respectively, in the absence of uncoupler.

After removal of uncoupler from the cells, the onset of precursor processing is rapid. The immunoprecipitate obtained after a 2 min chase in the absence of DNP (lane 5) contains a small amount of M_r 70000 (mature) subunit. Although it proved difficult to achieve complete resolution of the precursor and mature forms, even on the 6% (w/v) gel used in this experiment, it was estimated that the half-life of the large subunit precursor is between 5 and 7.5 min. Longer chase

Fig.3.6 Kinetics Of Processing Of The Large Subunit Of Succinate Dehydrogenase After Reversal Of Precursor Accumulation

PK-15 cells were incubated with [^{35}S]methionine in the absence or presence of 2mM DNP, as before. After pulse-labelling in the presence of DNP, a set of dishes was utilised to perform chases of increasing duration in the absence of uncoupler. After preparation of cell lysates at the appropriate time points, immunoprecipitation was performed using non-immune or anti-L serum. The resulting immunoprecipitates were resolved by SDS/polyacrylamide electrophoresis on a 6% (w/v) slab gel and visualised by fluorography. Lane 1, ^{125}I-labelled low M_r markers; lane 2, pulse (no uncoupler), non-immune serum; lane 3, pulse (no uncoupler), anti-L serum; lane 4, pulse + 2mM DNP, anti-L serum; lanes 5-10, as 4, but with 2 min, 5 min, 7.5 min, 10 min, 15 min and 30 min chase, respectively.


M_r x 10^{-3}
67
45
Pre-L
L
1
2
3
4
5
6
7
8
9
10

periods (lanes 9 and 10) revealed that processing was complete after 15 min.

c) Stability Of The Accumulated Precursor To The Large Subunit Of Succinate Dehydrogenase

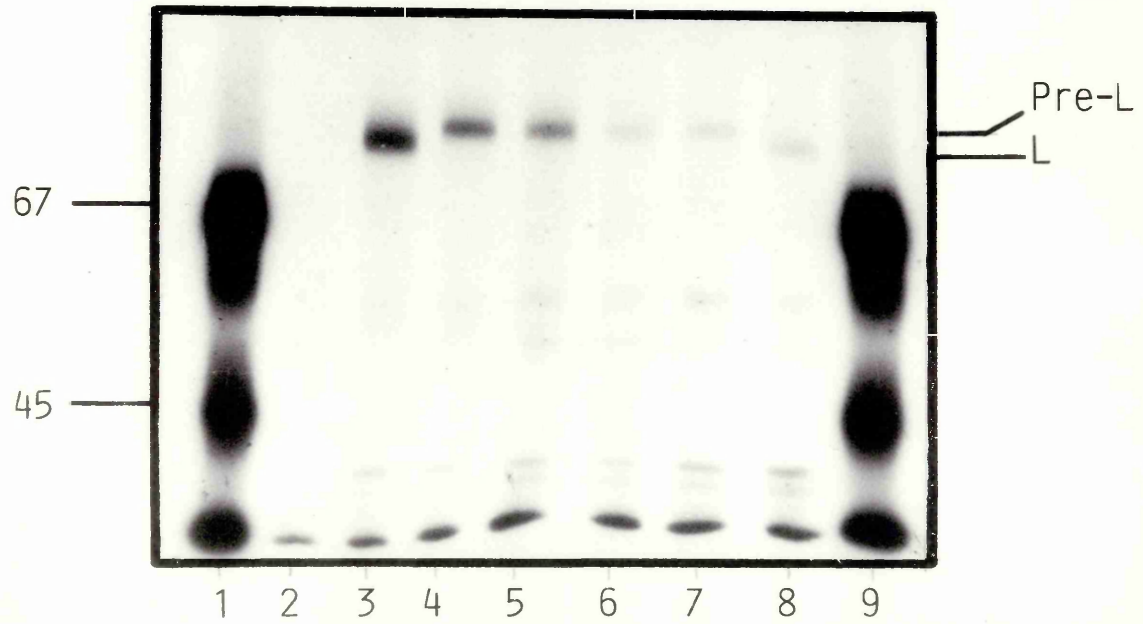
To monitor stability of the large subunit precursor accumulated during the pulse-label, a chase was performed for different time periods in the presence of uncoupler (Fig.3.7). Lane 4 shows the precursor recovered after a 4h pulse with 2mM DNP, while lanes 5-7 show the precursor recovered after a 4h pulse with uncoupler followed by a 1, 3 and 5h chase, respectively, also in the presence of uncoupler. Although the intensity of the precursor band decreases over this time period, a substantial proportion remains detectable even after a 5h chase. When the 5h chase in DNP-containing medium was followed by a 45 min chase in the absence of uncoupler, complete conversion of precursor to mature form was again observed (lane 8).

3.4 DISCUSSION

The data presented in Fig.3.1 establishes that the minor components present in the purified SDH preparation cross-react specifically with antiserum raised against the large subunit excised from an SDS/polyacrylamide gel. These species are most probably proteolytic fragments of the M_r 70000 polypeptide generated during the isolation procedure. Similar immunologically-reactive fragments of the large subunit can be detected after limited proteolysis of sub-

Fig.3.7 Stability Of The Accumulated Precursor To The Large Subunit Of Succinate Dehydrogenase

PK-15 cells were pulsed for 4h with [^{35}S]methionine in the absence or presence of 2mM DNP and then chased in the presence of DNP for the times indicated. In addition, cells which had been subjected to a 5h chase in the presence of DNP were incubated for a further 45 min in medium minus uncoupler. Immunoprecipitates were obtained from radiolabelled cell lysates and analysed as in Fig.3.6. Lanes 1 and 9, ^{125}I-labelled low M_r markers; lane 2, pulse (no uncoupler), non-immune serum; lane 3, pulse (no uncoupler), anti-L serum; lane 4, pulse + 2mM DNP; lanes 5-7, same as 4, but with 1h, 3h, and 5h chases, respectively; lane 8, same as 7, but with additional 45 min chase minus uncoupler.



M_r x 10^{-3}
67
45
Pre-L
L
1
2
3
4
5
6
7
8
9

mitochondrial particles in experiments designed to probe the organisation of SDH within the lipid bilayer (see Chapter Four).

Antiserum was produced also to the small subunit of SDH isolated in a similar manner. The weak cross-reactivity of anti-S serum with the large subunit may reflect genuine immunological similarity between the M_r 70000 and M_r 27000 subunits. This situation is not entirely unlikely, since both polypeptides contain sequences which participate in the formation of iron-sulphur clusters in the holoenzyme. However, the possibility that the material used for production of small subunit antiserum contained a M_r 27000 large subunit fragment cannot be excluded.

Several points have emerged from the immune blot analyses presented in this chapter. Firstly, immune blotting can be used to monitor potential loss of a polypeptide by proteolysis during its isolation. Secondly, the ability to detect proteolytic fragments in pure preparations offers an alternative criterion by which to assess the homogeneity of a purified protein. Clearly, the purity of the preparation used for immunisation in this case was greater than the original estimate. Finally, the cross-reactivity exhibited by the various antisera to SDH with the corresponding parent antigens from other tissues and species illustrates a potential immunologically-based method of identifying equivalent proteins in closely or even distantly-related sources.

The energy-dependent nature of the import of cytoplasmically-synthesised mitochondrial polypeptides was exploited to accumulate newly-synthesised forms of the subunits of succinate dehydrogenase in cultured mammalian cells. Fig.3.4 shows that when Buffalo rat liver (BRL) cells are incubated with [^{35}S]methionine and uncouplers of

oxidative phosphorylation, higher M_r precursor forms of both subunits can be immunoprecipitated. The precursors to the large and small subunits exhibit M_r values which are approximately 1-2000 and 4-5000 higher than the corresponding mature forms. The data for the precursor to the large subunit is in agreement with the estimate published by Ono and Tuboi (1986) during the latter stages of this study. These workers detected a large subunit precursor either *in vitro*, using rat liver mRNA to direct a cell-free translation system, or *in vivo* in ascites hepatoma cells. The data concerning the biosynthesis of the small subunit provides the first demonstration that the M_r 27000 polypeptide is also synthesised as a higher M_r precursor.

The existence of higher M_r precursor forms of the components of SDH in PK-15 cells was also demonstrated (Fig.3.5). The M_r values of the precursor forms in this cell line were comparable to the corresponding precursors in BRL cells. An interesting observation in the PK-15 cell line is that when cells are pulse-labelled with $[^{35}S]$methionine in the presence of 10μM FCCP, both precursor and mature forms of each subunit are detectable, indicating that translocation and processing are only partially inhibited in this case.

The reversible nature of uncoupler-induced precursor accumulation was demonstrated in experiments where complete conversion of precursor to mature form was observed after a 45 min chase in the absence of uncoupler. Chases of shorter duration revealed that onset of precursor processing after uncoupler removal is rapid, and that processing occurs with an estimated half-life of 5-7.5 min. Similar half-lives have been reported for other mammalian mitochondrial polypeptide precursors (Fenton *et al*., 1984; Mori *et al*., 1981; Raymond and Shore,

1981).

A noteable feature of the pig kidney large subunit precursor is its stability when import into the mitochondrion is inhibited. An appreciable amount of this polypeptide persists for at least 5h after synthesis. Furthermore, the accumulated precursor remains in an import-competent form, since processing is still observed when PK-15 cells are reintroduced to uncoupler-free medium after a 5h chase plus uncoupler. Similar long term stability has been reported for methylmalonyl-CoA mutase (Fenton et al., 1984).

What is the basis for this long term stability? Although the possibility exists that DNP acts on the cells to inhibit general proteolysis, this explanation is not favoured for two reasons. Firstly, pre-aspartate aminotransferase exhibits marked instability, being rapidly destroyed with a half-life of approximately 5 min when the accumulated precursor is chased in the presence of uncoupler (Jaussi et al., 1982). Secondly, in an experiment analogous to that shown in Fig.3.7, the precursors to the three component enzymes of the mitochondrial 2-oxoglutarate dehydrogenase complex were found to exhibit differing stabilities (Hunter, 1985). Thus, we propose that the observed long term stability is an inherent property of certain precursors, rather than a reflection of the experimental conditions employed.

CHAPTER FOUR

TOPOGRAPHICAL STUDIES ON SUCCINATE DEHYDROGENASE

4.1 INTRODUCTION

Although succinate dehydrogenase activity has traditionally been regarded as an enzymatic marker for the mitochondrial inner membrane (Ernster and Kuylenstierna, 1970), several major lines of evidence suggest that SDH is not completely buried in the inner membrane, but rather displays an asymmetric distribution with respect to the lipid bilayer.

Firstly, submitochondrial particles (everted vesicles, matrix side outermost) catalyse antimycin-insensitive electron transfer between succinate dehydrogenase and the membrane-impermeant anion ferricyanide, whereas intact mitochondria (outer membrane removed) do not (Klingenberg and Buchholz, 1970). Secondly, Merli *et al*. (1979) have shown that antiserum raised to the SDH holoenzyme is a potent inhibitor of succinate dehydrogenase activity in submitochondrial particles, but not in intact mitochondria. Moreover, when submitochondrial particles or intact mitochondria are modified with the membrane impermeant, protein-modifying reagent [^{35}S]diazobenzene-sulphonate, the large subunit of SDH is labelled only when the modification is performed from the matrix side of the inner membrane. Thirdly, by using radiolabelled (arylazido)phospholipids containing a photoactive group in the fatty acid or head group region of the molecule, Girdlestone *et al*. (1981) were able to investigate the interaction of purified succinate dehydrogenase or complex II with lipids by incubating the enzyme with lipid vesicles containing either probe, and effecting covalent cross-linking of protein with the lipid analogues by UV irradiation. The results of these experimemts led Girdlestone and coworkers to propose that the small subunit of SDH is

partially embedded in the lipid bilayer, whereas the large subunit is bound predominantly through electrostatic interactions with the membrane surface. Taken together, the above data strongly indicate that succinate dehydrogenase is located both functionally and physically on the matrix side of the mitochondrial inner membrane.

With the availability of high-quality antisera to the bovine heart SDH holoenzyme and its constituent large and small subunits, the opportunity existed to investigate further the topographical arrangement of this enzyme using an approach which had not been exploited in this context, i.e. protease treatment of the cytoplasmic or matrix face of the mitochondrial inner membrane followed by detection of protease-resistant, membrane-associated SDH fragments by immune blotting. This chapter describes the insights gained into the topography of succinate dehydrogenase using such an approach.

4.2 METHODS

4.2.1 PROTEASE TREATMENT OF FRESHLY-ISOLATED OR FREEZE-THAWED BOVINE HEART MITOCHONDRIA

Portions of a 4mg/ml solution of bovine heart mitochondria (prepared as described in section 2.2.7d) were incubated for 60 min at 30°C with α-chymotrypsin or trypsin at 4% (w/w) protease: mitochondrial protein ratio. A control incubation (no protease addition) was performed in parallel. Samples (1mg) were removed from each incubation at 0, 15, 30 and 60 min, and these were dissolved in an equal volume of hot (70°C) Laemmli sample buffer (section 2.2.3b) containing 10mM DTT. The dissolved samples were boiled for 5 min before analysis by SDS/polyacrylamide gel electrophoresis and immune

blotting using anti-SDH serum.

An identical protocol was followed for protease treatment of mitochondria which had been frozen at -80°C and subsequently thawed.

4.2.2 PROTEASE TREATMENT OF BOVINE HEART SUBMITOCHONDRIAL PARTICLES

Bovine heart submitochondrial particles, prepared as described in section 2.2.7e, were subjected to incubation with single or multiple additions of a range of proteases, including α-chymotrypsin, trypsin, papain and protease K. Incubations were performed at 30°C or 37°C, for periods of up to 90 min. The specific conditions used in each experiment are detailed in the appropriate figure legend and in section 4.3.

In some cases, proteolysis was terminated by mixing samples with 0.33 volumes of 2x Laemmli sample buffer (section 2.2.3b) containing 20mM DTT and 0.8mg/ml leupeptin (for inactivation of papain) or 2mM PMSF (for inactivation of α-chymotrypsin, trypsin or protease K). After 5 min at room temperature the samples were boiled for 5 min before analysis by SDS/polyacrylamide gel electrophoresis and immune blotting. Alternatively, aliquots of protease-treated submitochondrial particles were diluted with 3 volumes of ice-cold 50mM sodium phosphate, pH 7.5, 0.15M NaCl, containing 0.8mg/ml leupeptin or 2mM PMSF. Subsequent manipulations were performed at 4°C. The samples were centrifuged at 27000xg for 20 min then the pelleted membrane fractions were resuspended in 0.5ml of buffer containing protease inhibitor and recentrifuged as above. The wash procedure was performed once more with buffer, once with distilled water, and the resulting pellets were dissolved in Laemmli sample buffer (section 2.2.3) containing 10mM DTT

by boiling for 5 min.

Where double digestion experiments using papain and -chymotrypsin were performed, the first digestion was terminated before addition of the second protease to the sample. Papain was inactivated by adding a 5-fold excess (by weight) of leupeptin from a 1mg/ml aqueous solution. α-chymotrypsin was inhibited by adding PMSF from a 200mM solution in propan-2-ol to a final concentration of 2mM. After addition of protease inhibitors, samples were incubated for 5 min at room temperature before further manipulations.

4.3 RESULTS

4.3.1 PROTEASE TREATMENT OF FRESHLY ISOLATED AND FREEZE-THAWED BOVINE HEART MITOCHONDRIA

It is well recognised that in preparations of freshly-isolated bovine heart mitochondria, the cytoplasmic surface of the inner membrane is exposed to labelling agents such as lactoperoxidase, suggesting that the outer membrane has been removed by the isolation procedure (Boxer, 1975). Therefore, by applying the protease treatment/immune blotting procedure described above to fresh mitochondria, it was possible to investigate the existence of exposed regions of the subunits of SDH on the cytoplasmic surface of the inner membrane.

Fig.4.1A shows the results of incubation of freshly-isolated bovine heart mitochondria at 30°C for up to 60 min in the absence of protease (lanes 2-5), and after addition of 4% (w/w) α-chymotrypsin (lanes 6-9) or trypsin (lanes 10-13), as analysed by immune blotting using anti-SDH serum. Inspection of this figure reveals that in the

Fig.4.1 Immune Blot Analysis Of The Effect Of Proteases On SDH In Bovine Heart Mitochondria

Portions of freshly isolated (A) or freeze-thawed (B) bovine heart mitochondria were incubated for 60min at 30°C with α-chymotrypsin or trypsin (4% (w/w) protease:mitochondrial protein), or with no protease addition (control incubation). Samples were removed from each incubation at the indicated times, proteolysis was terminated, and the samples were subjected to electrophoresis on 10% (w/v) SDS/polyacrylamide slab gels. Resolved polypeptides were then electrophoresed onto nitrocellulose paper for detection of immunoreactive species using anti-SDH serum. Panels A and B; lanes 1 and 14, ^{125}I-labelled low M_r markers; lanes 2-5, control samples; lanes 6-9, digestion with α-chymotrypsin; lanes 10-13, digestion with trypsin. 40μg of protein was loaded in lanes 2-13 inclusive.

A.

Control α-CHYMO Trypsin

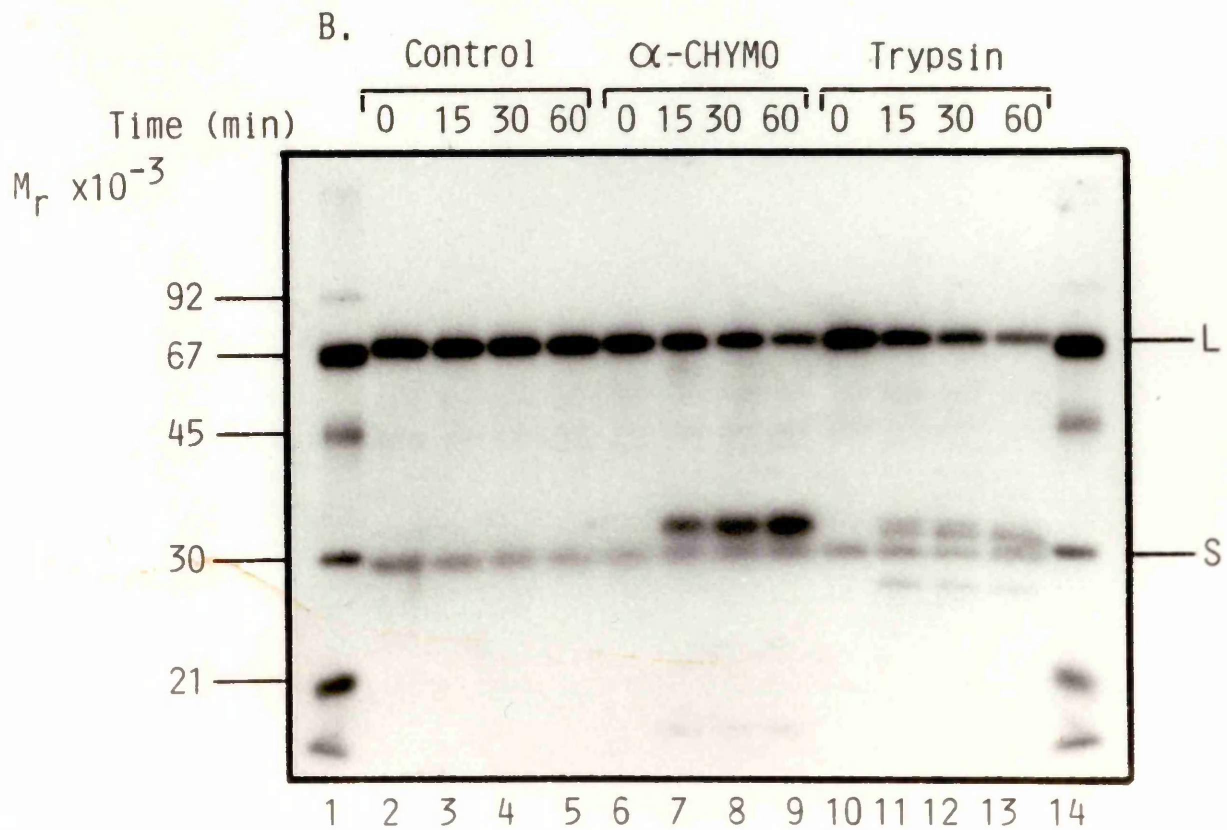
Time (min) 0 15 30 60 0 15 30 60 0 15 30 60

$M_r \times 10^{-3}$

92
67
45
30
21

L
S

1 2 3 4 5 6 7 8 9 10 11 12 13 14



control incubation, neither the large nor small subunit of SDH is susceptible to degradation, indicating that the enzyme is stable under the conditions employed. Inclusion of either α-chymotrypsin or trypsin during the incubation had no effect on the intensity of the bands corresponding to the large and small subunits of SDH. This observation suggested that neither subunit is exposed on the cytoplasmic face of the inner membrane.

To eliminate the possibility that SDH in its membrane environment is inherently resistant to proteolysis under the conditions described above, the experiment was repeated using mitochondria which had been frozen at -80°C and then thawed, since freeze-thawing is known to result in fracturing of the inner membrane (Pettit *et al.*, 1978). Fig.4.1B (lanes 6-9) illustrates that when freeze-thawed mitochondria are incubated with α-chymotrypsin for 60 min at 30°C, degradation can indeed be observed. Diminution of the band corresponding to the large subunit was paralleled by the appearance of an immunoreactive species of M_r approx. 32000, i.e. a large subunit proteolytic fragment. In contrast, there was little discernible decrease in the intensity of the band corresponding to the small subunit. When freeze-thawed mitochondria were similarly incubated with trypsin (lanes 10-13), degradation of both subunits of SDH was observed, with the concomitant appearance of immunoreactive species with M_r values of approx. 34000, 32000 and 24000, respectively. Although the two larger species can be identified as large subunit fragments, the 24000-M_r species may have been derived from either subunit. However, on the basis of the above observations, protease digestion experiments were subsequently performed using submitochondrial particles.

4.3.2 PROTEASE TREATMENT OF BOVINE HEART SUBMITOCHONDRIAL PARTICLES

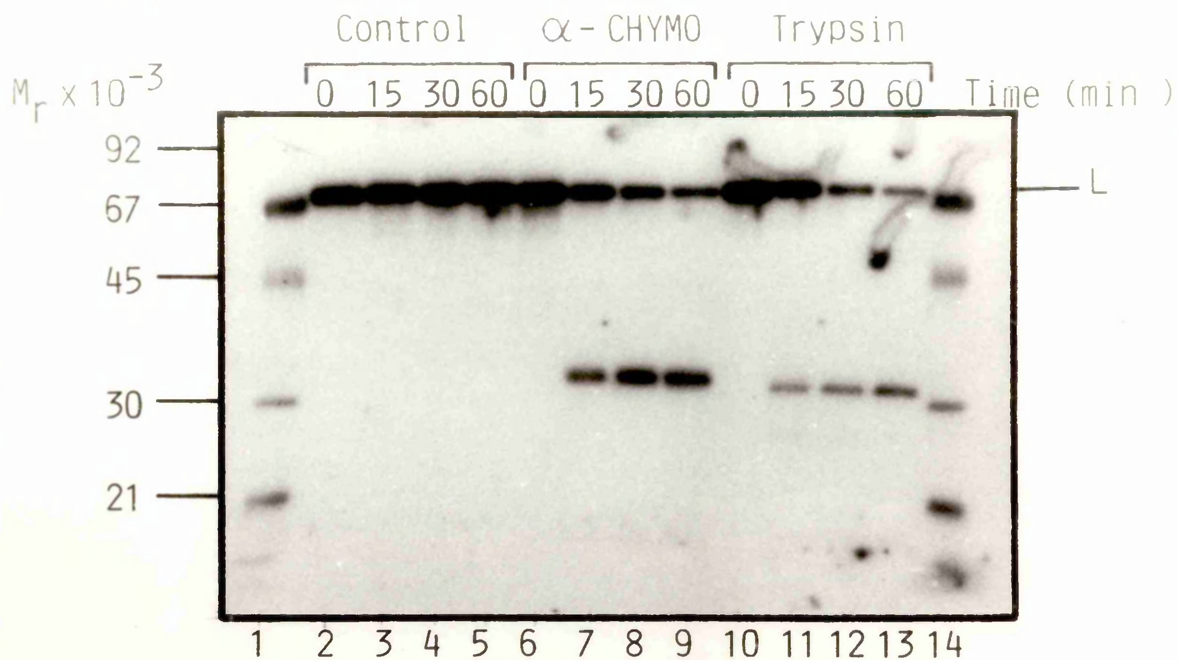
Bovine heart submitochondrial particles (SMPs), generated by sonication of intact mitochondria, are predominantly vesicles of inner membrane with an inverted orientation (Godinot and Gautheron, 1979). Hence, the exposure of SDH on the matrix side of the mitochondrial inner membrane was investigated by immune blot analysis of protease-treated SMPs.

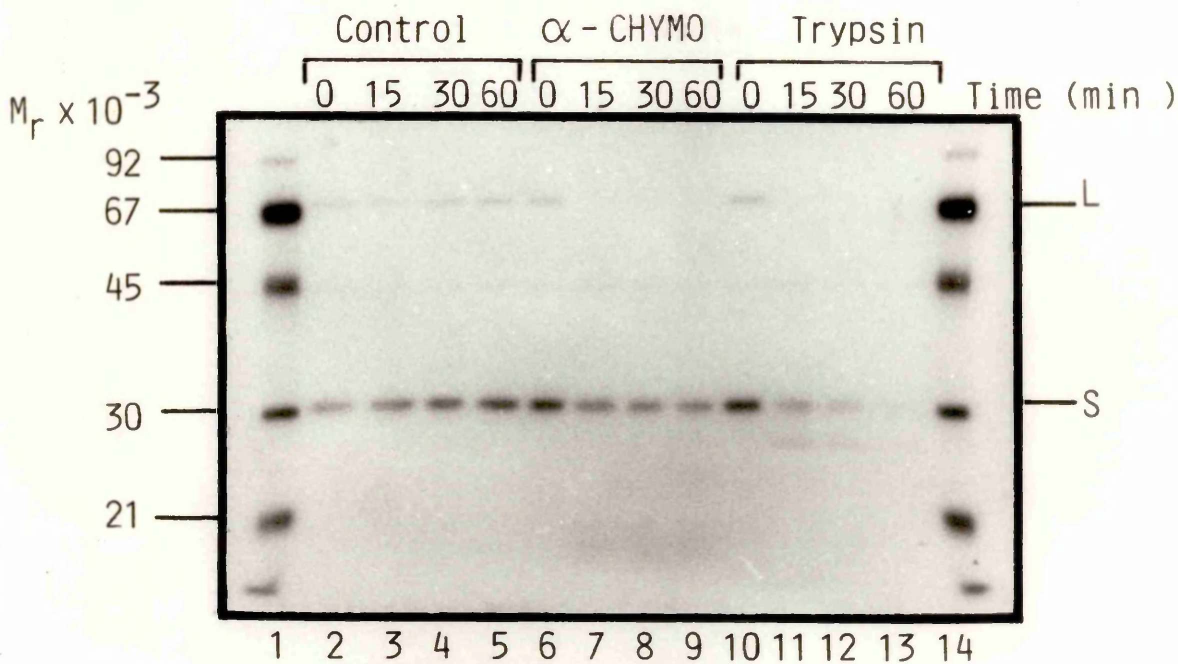
Submitochondrial particles were incubated at $30^{\circ}C$ in the absence of protease, and with 4% (w/w) α-chymotrypsin or trypsin for up to 60 min. Fig.4.2 illustrates the immune blot patterns obtained when control or protease-treated particles were probed with subunit-specific anti-L serum (panel A) or anti-S serum (panel B). The data indicates that in the absence of protease both subunits of SDH were stable during the incubation (lanes 2-5), whereas in the presence of α-chymotrypsin (panel A, lanes 6-9) or trypsin (lanes 10-13) the large subunit was degraded with the parallel appearance of a fragment of M_r approx. 32000. The tryptic digests contained in addition a fragment of M_r approx. 24000, which was subsequently shown to be soluble rather than membrane-associated (see Fig.4.3).

The immune blot obtained using anti-S serum reveals that treatment with α-chymotrypsin resulted in a slight diminution in the intensity of the band corresponding to the small subunit, although a chymotryptic small subunit fragment was not detectable (panel B, lanes 6-9). Incubation with trypsin resulted in degradation of the small subunit (lanes 10-13), with the parallel appearance of a 24000-M_r immunoreactive species. Although anti-S serum exhibits weak cross-reactivity with the large subunit (section 3.3.1 and this figure),

Fig.4.2 The Effect Of Proteases On The Subunits Of SDH In Bovine Heart Submitochondrial Particles

Bovine heart submitochondrial particles were incubated for 60 min at 30°C in the absence of protease, or after addition of α-chymotrypsin or trypsin (4% (w/w) protease:particle protein ratio). In each case, samples were removed at 0, 15, 30, and 60 min, mixed with 0.33 volumes of 2x Laemmli sample buffer (section 2.2.3b) containing 10mM DTT, then boiled for 5 min. The samples were subjected to electrophoresis on 10% (w/v) SDS/polyacrylamide slab gels followed by immune blotting using subunit-specific anti-L (A) or anti-S (B) sera. Panels A and B; lanes 1 and 14, ^{125}I-labelled low M_r markers; lanes 2-5, control samples; lanes 6-9, digestion with α-chymotrypsin; lanes 10-13, digestion with trypsin. 40µg of protein was loaded in lanes 2-13 inclusive.


A.
Control
α - CHYMO
Trypsin
$M_r \times 10^{-3}$
0 15 30 60 0 15 30 60 0 15 30 60 Time (min)
92
67
45
30
21
L
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B.
Control
α - CHYMO
Trypsin
$M_r \times 10^{-3}$
0 15 30 60 0 15 30 60 0 15 30 60 Time (min)
92
67
45
30
21
L
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14

this 24000-M_r tryptic fragment is most probably derived from the small subunit, since even the levels of intact large subunit in the trypsin-treated samples are undetectable using anti-S serum.

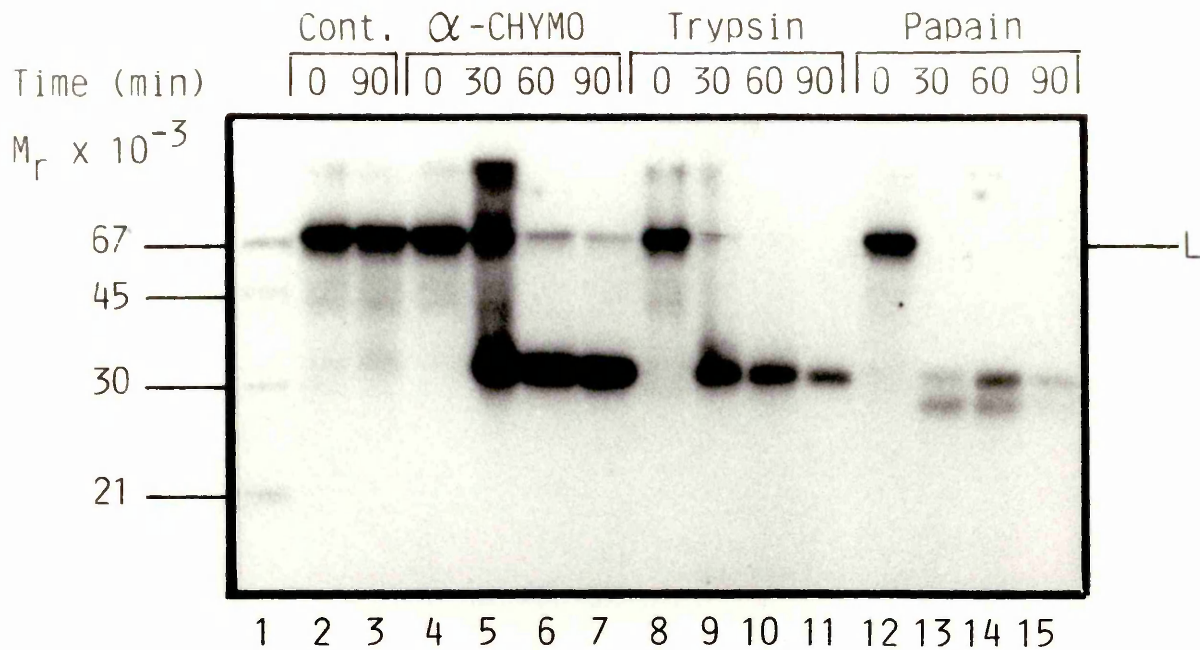
To test whether the proteolytic fragments observed in Fig.4.2 were membrane-associated, protease-treated SMPs were washed with buffer containing 0.15M NaCl before immune blot analysis. Fig.4.3 illustrates the patterns obtained when SMPs which had been incubated at 30°C for up to 90 min with 4% (w/w) α-chymotrypsin (lanes 4-7), trypsin (lanes 8-11) or papain (lanes 12-15) were salt-washed and analysed by immune blotting using anti-L (panel A) or anti-S (panel B) serum. The blot in panel A reveals that the previously observed chymotryptic and tryptic fragments (M_r approx. 32000) remained membrane-associated after the salt wash treatment. Incubation with papain generated two membrane-associated fragments; one exhibited an identical electrophoretic mobility to the 32000-M_r tryptic fragment, while the second had an M_r value of 27000. The latter fragment was not recognised by anti-S serum.

The previously observed 24000-M_r tryptic fragment (Fig.4.3B, lanes 10-13) was not detectable after the protease-treated membranes were salt washed (lanes 8-11). This observation suggested that this species was not a _bona fide_ membrane-associated fragment. The small subunit was also susceptible to degradation by papain, although as with trypsin, no proteolytic fragment was readily detectable.

It was also found that treatment of SMPs with 4% (w/w) protease K at 30°C for 30 min generated two large subunit fragments, with M_r values of approx. 32000 and 27000, similar to the doublet obtained by papain digestion. This was paralleled by virtually complete cleavage of the intact large subunit (Fig.4.4). Both protease K large subunit

Fig.4.3 Immune Blot Analysis Of Salt-Washed, Protease-Treated Submitochondrial Particles Using Anti-L And Anti-S Sera

Bovine heart submitochondrial particles were incubated at 30°C in the absence of protease, or after addition of α-chymotrypsin, trypsin or papain (4% (w/w) protease:particle protein). In each case, samples (1mg particle protein) were removed at 0, 30, 60 and 90 min, and mixed with 3 volumes of ice-cold 50mM sodium phosphate, pH 7.5, 0.15M NaCl containing 2mM PMSF or 0.8μg/μl leupeptin. The samples were then processed as described in section 4.2.2, resolved by electrophoresis on 15% (w/v) SDS/polyacrylamide slab gels (section 2.2.3), and analysed by immune blotting (section 2.2.8a) using subunit-specific anti-L (A) or anti-S (B) serum. Panels A and B; lanes 2 and 3, control samples; lanes 4-7, digestion with α-chymotrypsin; lanes 8-11, digestion with trypsin; lanes 12-15, digestion with papain. Lanes 1 (A and B) and 16 (B) contain ^{125}I-labelled low M_r markers.


A.
Cont.
α-CHYMO
Trypsin
Papain
Time (min)
0 90
0 30 60 90
0 30 60 90
0 30 60 90
$M_r \times 10^{-3}$
67
45
30
21
L
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

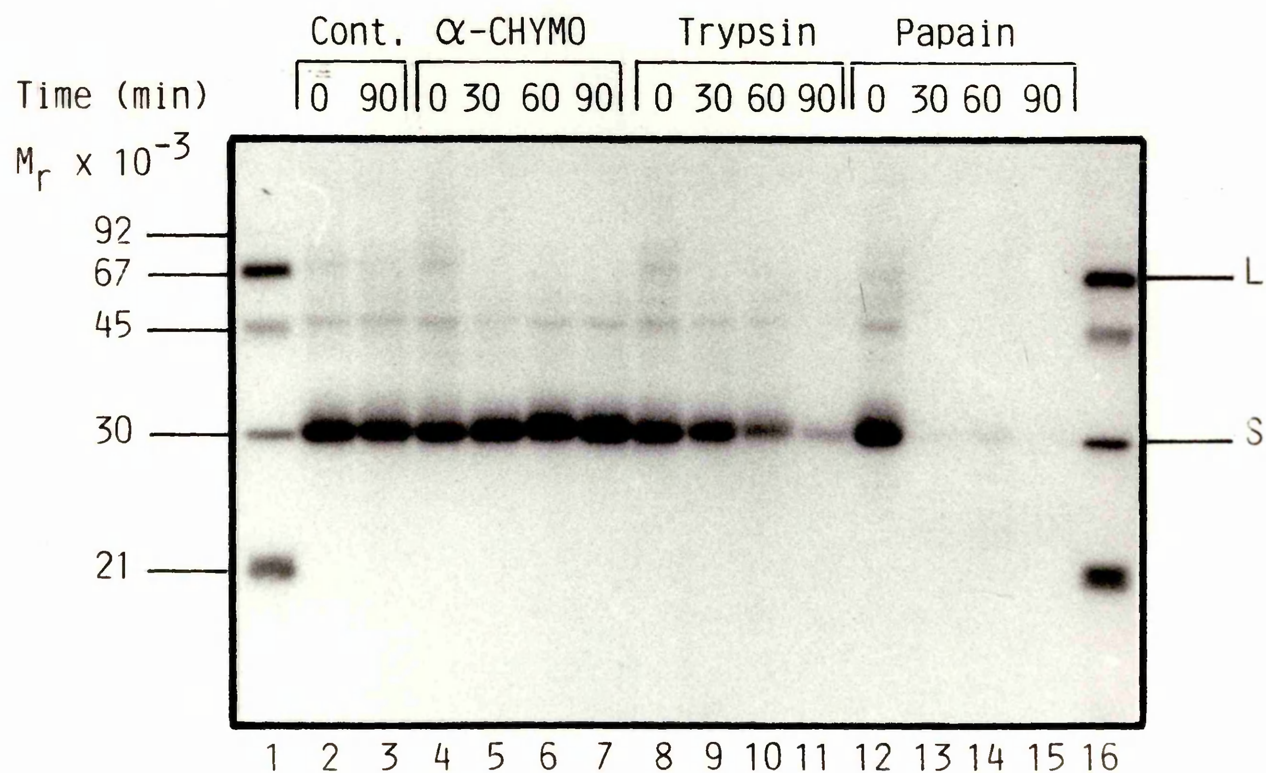
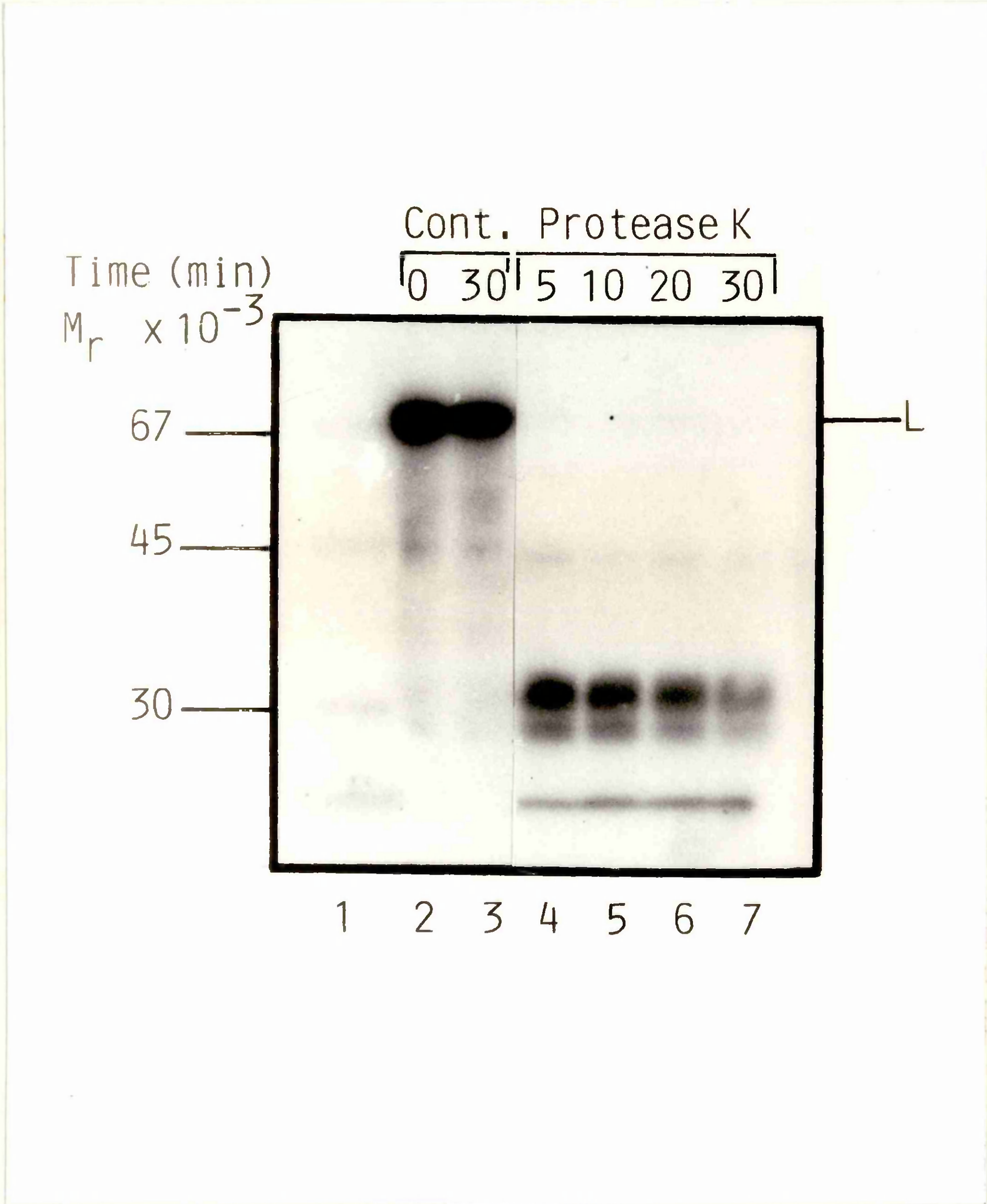

B.
Cont.
α-CHYMO
Trypsin
Papain
Time (min)
0 90
0 30 60 90
0 30 60 90
0 30 60 90
$M_r \times 10^{-3}$
92
67
45
30
21
L
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig.4.4 Immune Blot Analysis Of Protease K-Treated Submitochondrial Particles Using Anti-L Serum

Bovine heart submitochondrial particles were incubated for 30 min at 30°C in the absence of protease or after addition of protease K (4% (w/w) protease:particle protein). Samples (100μg particle protein) were removed at 5, 10, 20 and 30 min, mixed with 0.33 volumes of 2x Laemmli sample buffer containing 10mM DTT and 2mM PMSF, and boiled for 5 min. The samples were then subjected to electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel followed by immune blot analysis using anti-L serum. Lane 1, ^{125}I-labelled low M_r markers; lanes 2 and 3, control samples (0 and 30 min); lanes 4-7, digestion with protease K.


Cont.
Protease K
Time (min)
0
30
5
10
20
30
M_r x 10^{-3}
67
45
30
L
1
2
3
4
5
6
7

fragments were subsequently found to remain membrane-associated after salt washing (data not shown).

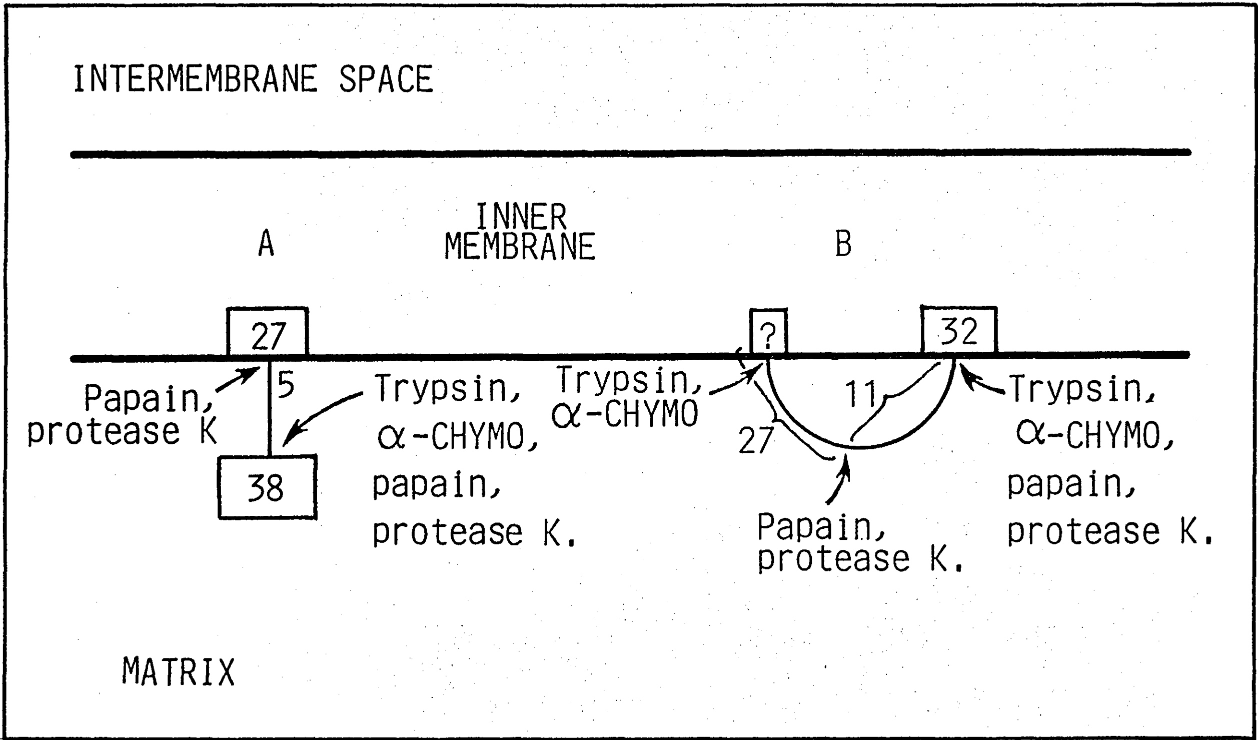
The existence of two membrane-associated large subunit fragments is consistent with two alternative models for the arrangement of the SDH large subunit in the mitochondrial inner membrane. These alternatives are depicted in Fig.4.5. In one case (model A), the large subunit is associated with the lipid bilayer via a single domain. Proteolytic cleavage by α-chymotrypsin, trypsin, papain or protease K generates a membrane-associated 32000-M_r fragment. In addition, cleavage with papain or protease K generates in some cases a 27000-M_r membrane-associated fragment which is derived from the above 32000-M_r species by removal of an additional 5000-M_r peptide.

Thus, model A predicts that, if SMPs are treated with α-chymotrypsin to completely cleave the intact large subunit and generate the 32000-M_r large subunit fragment, subsequent incubation with papain or protease K (after inactivation of α-chymotrypsin) will result in appearance of the 27000-M_r large subunit fragment. Conversely, if the order of protease addition is reversed, the 27000-M_r papain (or protease K) fragment will remain after incubation with -chymotrypsin.

The alternative model (B in Fig.4.5) proposes that the large subunit polypeptide is associated with the bilayer via two distinct regions; a 32000-M_r region which is generated by cleavage with α-chymotrypsin, trypsin, papain or protease K, and a second region, of undetermined size, which is detected as a 27000-M_r membrane-associated papain or protease K fragment, but is not detectable in α-chymotrypsin- or trypsin-treated SMPs. In this case, the model predicts that when α-chymotrypsin treatment is followed by treatment with papain (or protease K), there will be no change observed in the immune

Fig.4.5 Alternative Models For The Arrangement Of The Large Subunit Of SDH In The Mitochondrial Inner Membrane

Schematic representation, based on protease digestion/immune blotting data. The arrows (⟶) indicate sites of proteolytic cleavage. The numerical values refer to the sizes of the different proteolytic fragments, given as $M_r \times 10^{-3}$. In model A, one region of the large subunit is membrane-associated; in B, the polypeptide has an additional membrane-associated domain, of unknown size. α-CHYMO; α-chymotrypsin.


INTERMEMBRANE SPACE
A
INNER
MEMBRANE
B
27
?
32
5
11
27
38
Papain,
protease K
Trypsin,
α-CHYMO,
papain,
protease K.
Trypsin,
α-CHYMO
Papain,
protease K.
Trypsin,
α-CHYMO,
papain,
protease K.
MATRIX

blot pattern, i.e. the 32000-M_r chymotryptic fragment will be present before and after papain treatment. Also, if the order of protease addition is reversed, the 27000-M_r fragment generated by papain (or protease K) would disappear since it will be degraded further by α-chymotrypsin to an undetectable species.

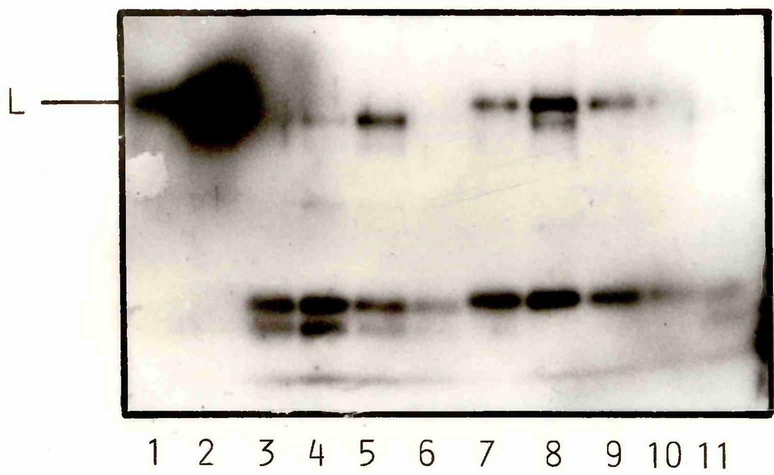
Fig.4.6 illustrates the pattern of large subunit fragments obtained when submitochondrial particles were treated successively with papain and α-chymotrypsin, in both possible orders of protease addition. Incubation at 37°C for 30 min with 4% (w/w) papain resulted in complete disappearance of the intact large subunit, and, as expected, appearance of 32000-M_r and 27000-M_r large subunit fragments (lane 3). These two fragments remained detectable in papain-treated vesicles which had been subjected to a further 20 min incubation after the addition of leupeptin (lane 4).

When digestion with papain was followed by incubation with 4% (w/w) α-chymotrypsin for 10 min (lane 5), or for a further 10 min after addition of a second identical aliquot of α-chymotrypsin (lane 6), the ratio of 32000-M_r fragment : 27000-M_r fragment was increased. This result, which was confirmed by densitometric scanning of the autoradiograph shown in Fig.4.6, indicated that α-chymotrypsin caused preferential degradation of the 27000-M_r polypeptide in papain-treated submitochondrial particles.

Incubation with 4% (w/w) α-chymotrypsin at 37°C for 10 min resulted in partial degradation of the large subunit, with the concomitant appearance of a 32000-M_r species (lane 7). When the vesicles were subjected to one or two further identical additions of α-chymotrypsin and one or two subsequent 10 min incubations (lanes 8 and 9 respectively), there was little if any decrease in the ratio of

Fig.4.6 Effects Of Double Digestions Using Papain And -Chymotrypsin On The SDH Large Subunit In Submitochondrial Particles

Submitochondrial particles were incubated at 37°C in the absence of protease, or after addition of papain and α-chymotrypsin, as detailed below. Where double digestions were performed, the first protease was inactivated (see section 4.2.2) before addition of the second. At the end of the appropriate incubation, each sample was mixed with 3 volumes of ice-cold 50mM sodium phosphate, pH 7.5, 0.15M NaCl containing 2mM PMSF or 0.8μg/μl leupeptin. The samples were then processed as in section 4.2.2, resolved by electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel (section 2.2.3), and analysed by immune blotting (section 2.2.8a) using anti-L serum. Lanes 1 and 2, control samples (0 and 60 min); lane 3, digestion with 4% (w/w) papain for 30 min; lane 4, as 3, but sample incubated for additional 20 min after papain inactivation; lane 5, as 3, but with additional 10 min incubation with 4% (w/w) α-chymotrypsin; lane 6, as 5, but with subsequent 10 min incubation after a second addition of 4% (w/w) α-chymotrypsin ; lane 7, digestion with 4% (w/w) α-chymotrypsin for 10 min; lane 8, as 7, but with subsequent 10 min incubation after a second addition of 4% (w/w) α-chymotrypsin; lane 9, as 8, but with subsequent 10 min incubation after a third addition of 4% (w/w) α-chymotrypsin; lane 10, as 9, but sample incubated for a further 15 min after inactivation of α-chymotrypsin; lane 11, as 9, with additional 15 min incubation after adding 4% (w/w) papain.


L
1 2 3 4 5 6 7 8 9 10 11

$70000\text{-}M_r$: $32000\text{-}M_r$ polypeptides. When vesicles which had been incubated identically to those shown in lane 9 were subjected to a further 15 min incubation with 4% (w/w) papain, further degradation of the large subunit was observed, coupled with the appearance of a $27000\text{-}M_r$ species (lane 11). In a control incubation, in which α-chymotrypsin-treated vesicles were incubated for a further 15 min after the addition of PMSF, there was no degradation of the $32000\text{-}M_r$ fragment to the $27000\text{-}M_r$ species (lane 10), although the amount of protein in the final pellet was reduced relative to the sample in lane 9.

4.4 DISCUSSION

In this chapter, the topographical arrangement of succinate dehydrogenase within the mitochondrial inner membrame was investigated using an approach which combined protease treatment of either face of this lipid bilayer with subsequent detection of the resulting proteolytic fragments by immune blot analysis. Bovine heart mitochondria were used for these experiments, since antisera had been raised to the bovine heart enzyme, and also because the commonly used method of isolating these mitochondria (Smith, 1967) renders the cytoplasmic face of the inner membrane accessible to exogenously-added enzymes (Boxer, 1975).

When freshly-isolated mitochondria were treated with α-chymotrypsin or trypsin and then analysed by SDS/polyacrylamide gel electrophoresis and immune blotting, both subunits of succinate dehydrogenase were found to be resistant to proteolytic degradation. Although a means of detecting degradation of the outer face of the

inner membrane (e.g. an antiserum directed against a polypeptide which protrudes into the intermembrane space) was not available, a Coomassie blue-stained gel of the protease-treated samples revealed that limited proteolysis of many polypeptides had occurred. Moreover, when freeze-thawed mitochondria were subjected to protease treatment in an identical manner, degradation of both subunits of SDH was observed. These data are consistent with neither subunit being exposed at the cytoplasmic surface of the mitochondrial inner membrane.

Inverted inner membrane vesicles (submitochondrial particles), generated by sonicating isolated bovine heart mitochondria, were used to investigate the accessibility of succinate dehydrogenase to proteases added from the matrix side of the inner membrane.

Samples of protease-treated submitochondrial particles were processed in one of two ways. Using the simpler procedure, proteolysis was terminated by mixing samples with concentrated Laemmli sample buffer containing protease inhibitors and incubating at room temperature for 5 min prior to boiling. This method of sample treatment was advantageous since it was rapid, and allowed equal amounts of starting material from each sample to be loaded on an SDS/polyacrylamide gel; however, a major drawback was that membrane-associated SDH fragments could not be distinguished from soluble peptides, since in this case the material analysed contained the complete range of digestion products.

Alternatively, protease-treated samples were subjected to three washes with buffer containing protease inhibitor and 0.15M NaCl, and a final wash with distilled water before the final membrane pellets were dissolved in Laemmli sample buffer. This procedure permitted removal of loosely-associated soluble peptides from the protease-

treated vesicles, but loss of membrane during the repeated centrifugations was a factor which had to be taken into account.

Proteolytic degradation of succinate dehydrogenase in submitochondrial particles was monitored using subunit-specific antisera. A common effect of a range of proteases, including α-chymotrypsin, trypsin, papain, protease K (this study) and pronase and elastase (A. Phelps, personal communication) was the appearance of a large subunit fragment of M_r approx. 32000. In each case, this species was shown to be tightly bound to the inner membrane, since it was not removed by the salt wash procedure described above, and was stable while membrane integrity was preserved.

The generation of such a large subunit fragment, even by low-specificity proteases, is evidence of a membrane-associated 32000-M_r large subunit domain. This domain may be joined to the remainder of the large subunit by a protease-sensitive 'hinge' region.

The reproducible appearance of an additional membrane-associated 27000-M_r large subunit fragment in samples of papain- and protease K-treated vesicles can be rationalised in one of two ways (Fig.4.5). The smaller fragment may be derived from the larger by removal of an additional 5000-M_r peptide from the 32000-M_r membrane-associated domain (model A in figure). Alternatively, it may represent a separate membrane-associated region (model B). In the latter case, digestion with proteases such as α-chymotrypsin generates a second large subunit fragment which evades detection by immune blot analysis. Lack of detection of such a species can be explained by a) lack of reactivity towards anti-L serum; b) migration near or at the dye front during SDS/polyacrylamide gel electrophoresis and c) the 32000-M_r species seen in immune blots representing two different proteolytic fragments

of identical size.

The above models enable predictions to be made concerning the effect on the fragment pattern generated by one protease of subsequent digestion with a second protease. Accordingly, submitochondrial particles were treated either with papain then α-chymotrypsin or with α-chymotrypsin followed by papain. When papain-treated vesicles, devoid of intact large subunit, were incubated subsequently with α-chymotrypsin specific and preferential degradation of the 27000-M_r papain fragment was observed. This result argues for an arrangement of the type shown in Fig.4.5B, where the second chymotryptic fragment is smaller than both papain fragments.

When the proteases were added in reverse order, α-chymotrypsin generated the expected 32000-M_r fragment, but failed in this case to effect complete cleavage of the large subunit. Subsequent addition of papain caused more complete degradation of the large subunit, and appearance of a 27000-M_r fragment. However, since in this instance the 27000-M_r fragment may be derived from intact large subunit or the 32000-M_r chymotryptic fragment, the observed effect is not inconsistent with a double domain structure as proposed above.

Additional, indirect evidence exists concerning the nature of the 27000-M_r large subunit fragment. If this species arose through further proteolysis of the 32000-M_r fragment, a precursor-product relationship should exist between these two polypeptides. Such an effect was not observed during prolonged incubation of papain- or protease K-treated vesicles after generation of the pair of fragments. This finding also suggests that the 27000-M_r species is derived from a separate region of the intact large subunit.

Although the small subunit of succinate dehydrogenase was shown

to be susceptible to the action of several different proteases added from the matrix side of the inner membrane, no direct evidence of protease-resistant, membrane-associated regions of this molecule was obtained. However, a putative small subunit fragment corresponding to most of the intact polypeptide (M_r approx. 24000) was observed in trypsin-treated submitochondrial particles, but only in the absence of salt washing. This finding is consistent with an arrangement where the bulk of the small subunit protrudes from the inner membrane, and only a small portion of the polypeptide extends into the interior of the bilayer. In addition, the rate of small subunit degradation was generally observed to be slower than that of the large subunit. This may reflect protection of the small subunit by the M_r 70000 polypeptide.

CHAPTER FIVE

PURIFICATION OF
BRANCHED CHAIN 2-OXOACID DEHYDROGENASE COMPLEX
FROM BOVINE KIDNEY AND
CHARACTERISATION OF ANTISERA RAISED AGAINST THIS ENZYME

5.1 INTRODUCTION

As described in section 3.1, a requirement for the investigation of mitochondrial polypeptide biosynthesis is a high-quality antiserum to the component(s) of interest. Therefore, an early objective of this project was to obtain highly purified BCOAD complex which could be used for production of antisera to the complex.

It was decided to isolate the BCOAD complex from bovine kidney for two main reasons. Firstly, a bovine kidney (NBL-1) cell line was available which could be used for *in vivo* biosynthetic studies on the BCOAD complex, and which had already been successfully employed for similar studies on other mitochondrial polypeptides. Secondly, a new procedure (Lawson *et al*., 1983) had recently been published for the isolation of milligram quantities of high-purity enzyme from this tissue. This procedure had the advantage of being relatively rapid, since it dispensed with the need for prior stock-piling of mitochondria, a feature of an earlier purification procedure (Pettit *et al*., 1978).

The BCOAD complex isolated from bovine kidney was used for production of antiserum to the native complex. The E2 component of the BCOAD complex was purified by preparative SDS/polyacrylamide gel electrophoresis and used for immunisation purposes. This method of isolating E2 was advantageous since it circumvented the need to employ more cumbersome separation procedures, and afforded the opportunity to raise antibodies to the SDS-denatured E2 component. The latter point was of relevance since a previous case had been encountered where antibodies to the native 2-oxoglutarate dehydrogenase E2 subunit cross-reacted only weakly with pre-E2 when compared with antibodies

raised to denatured E2 (Hunter and Lindsay, 1986).

Latterly, it proved possible to obtain samples of purified BCOAD complex and the isolated E1 subcomplex from Dr. S.J. Yeaman (Dept. of Biochemistry, University of Newcastle upon Tyne). Antiserum was therefore also raised to the native E1 subcomplex of bovine kidney BCOAD.

This chapter deals with the purification of the BCOAD complex from bovine kidney and the production of antibodies to the native BCOAD complex (anti-BCOAD serum), the native E1 subcomplex (anti-E1 serum) and the denatured E2 subunit (anti-E2 serum). The characterisation of these antisera by immune blot analysis with their parent antigens and with cultured cell subfractions is also described.

5.2 METHODS

5.2.1 PURIFICATION OF BCOAD COMPLEX FROM BOVINE KIDNEY

The following buffers were prepared and cooled to $4^{o}C$:-

Buffer A; 50mM potassium phosphate, pH 7.5, 2mM EDTA, 3% (v/v) Triton X-100, 1mM benzamidine-HCl, 1mM PMSF, 0.1mM DTT.

Buffer B; 30mM potassium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT.

Buffer C; 30mM sodium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT.

Buffer D; 200mM potassium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT.

Buffer E; 350mM potassium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT.

Benzamidine-HCl, DTT and PMSF were added to the buffers

immediately before use from concentrated stock solutions.

Fresh bovine kidneys were transported on ice to the laboratory. All subsequent operations were performed at $4^{o}C$. Approximately 220g of cortex tissue was cut into small pieces and homogenised in a Moulinex blender for 5 min with two volumes of buffer A containing 0.05% (v/v) antifoam A. The resulting homogenate was mixed with an equal volume of buffer A and centrifuged at 15000xg for 26 min. The pH of the supernatant fluid was measured and, when necessary, adjusted to 7.3 with 10% (v/v) acetic acid.

0.12 volumes of 35% (w/v) polyethylene glycol (PEG) was then added and the mixture was stirred for 30 min. The resulting precipitate was collected by centrifugation at 15000xg for 30 min and resuspended in buffer B (0.33 initial volume) by homogenisation in a Potter-Elvenhejm teflon/glass homogeniser. The resuspended material was stirred for 30 min (PEG I) and then clarified by centrifugation at 19000xg for 27 min (PEG I clarified). 0.12 volumes of 35% (w/v) PEG was added to the clarified material, and the mixture was stirred for 30 min before the precipitate was collected by centrifugation at 15000xg for 30 min. The precipitate was resuspended in buffer B (0.05 initial volume), then homogenised, stirred (PEG II) and clarified (PEG II clarified) as above.

The material obtained in this manner was applied at a flow rate of 12ml/h to a column of Bio-Gel HTP (2.6 x 5.5cm) equilibrated in buffer B. The column was then washed at 24ml/h with buffer D until the absorbance at 280nm of the eluate was less than 0.01. BCOAD complex was eluted from the column with buffer E at a flow rate of 24ml/h. Fractions from the peak of BCOAD activity were centrifuged separately at 40000xg for 16h. The resulting pellets of purified BCOAD complex

were each resuspended in 1ml of buffer C. The resuspended material was stored at $-20^{\circ}C$ after addition of glycerol to 25% (v/v) final concentration.

5.2.2 PREPARATION OF ANTISERUM TO BOVINE KIDNEY BCOAD COMPLEX AND ITS E1 AND E2 COMPONENTS

Antisera to native BCOAD complex (anti-BCOAD serum) and the native E1 subcomplex (anti-E1 serum) were prepared by adopting the protocol described for the production of anti-SDH serum (section 3.2.1). Preparative SDS/polyacrylamide gel electrophoresis (section 2.2.3) was employed to resolve purified BCOAD complex into its three constituent polypeptides. Subunit-specific anti-E2 serum was prepared in an analogous manner to anti-L and anti-S sera (section 3.2.1), using the denatured E2 band excised from preparative gels.

5.2.3 INCUBATION OF PURIFIED BCOAD COMPLEX WITH NON-IMMUNE, ANTI-BCOAD, ANTI-E1 OR ANTI-E2 SERUM

Purified BCOAD complex (25mg/ml) was diluted to a concentration of 2.2 mg/ml with 50mM sodium phosphate buffer, pH 7.0, containing 12mg/ml BSA, 0.2mM TPP, 1mM $MgCl_2$, 2mM DTT, and 0.2mM EDTA (diluent buffer). 20μl (44μg) portions of enzyme diluted in this way were incubated for 18h at $4^{\circ}C$ with various volumes (4-40μl) of serum. In each case, the reaction was performed in a final volume of 220μl (final enzyme concentration 0.2μg/μl) by adding the appropriate volume of diluent buffer. After the incubation period, duplicate 10μg portions of enzyme from each tube were assayed for overall BCOAD

activity, as described in section 2.2.1. Results were expressed as a percentage of the activity of the enzyme after dilution to a final concentration of 0.2mg/ml with diluent buffer.

5.3 RESULTS

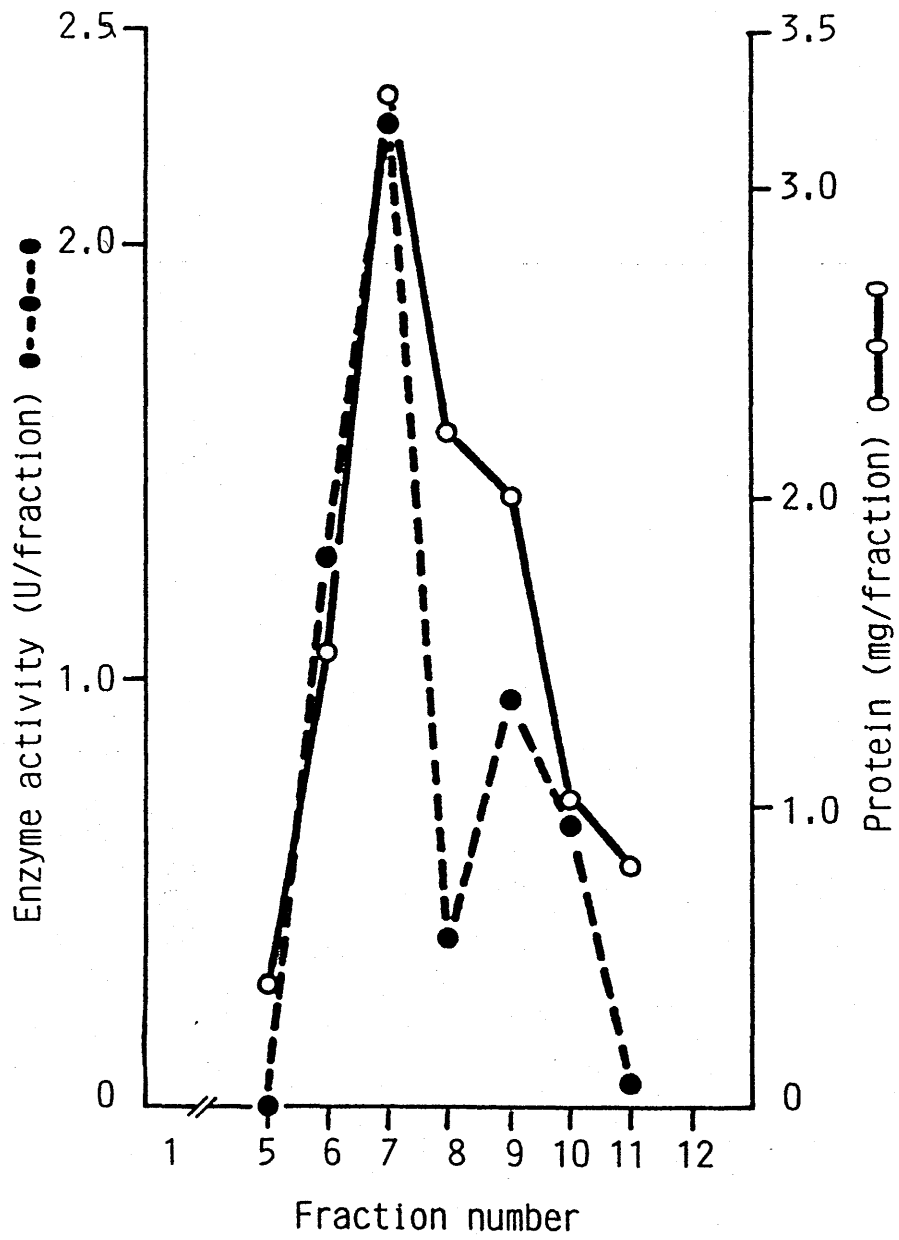
5.3.1 PURIFICATION OF BRANCHED CHAIN 2-OXOACID DEHYDROGENASE COMPLEX FROM BOVINE KIDNEY

The method of Lawson et al. (1983) was employed to purify the BCOAD complex from bovine kidney cortex. Fig.5.1 shows the result of chromatography of partially purified BCOAD complex (PEG II clarified material, section 5.2.1) on Bio-Gel HTP. The complex was bound relatively tightly by the hydroxylapatite matrix, remaining bound after washing of the column with buffer containing 200mM potassium phosphate (buffer D, section 5.2.1). The majority of contaminating polypeptides were eluted from the column using this buffer. The complex could, however, be eluted using buffer containing 350mM potassium phosphate (buffer E, section 5.2.1). Under these conditions, the enzymic activity eluted essentially as a single peak which correlated closely with the A_{280} profile of the eluate.

Fig.5.2 illustrates the polypeptide pattern of samples from each stage of a preparation which yielded high-purity enzyme, as analysed by SDS/polyacrylamide slab gel electrophoresis and Coomassie blue staining. It is apparent from this figure that the hydroxylapatite step results in extensive removal of contaminating polypeptides from the final product (e.g. compare lanes 6 and 7). The highly purified fractions contained three major bands. By comparison with the electrophoretic mobilities of a set of low M_r marker proteins, the apparent

Fig.5.1 <u>Chromatography Of Partially Purified Bovine Kidney BCOAD Complex On Bio-Gel HTP</u>

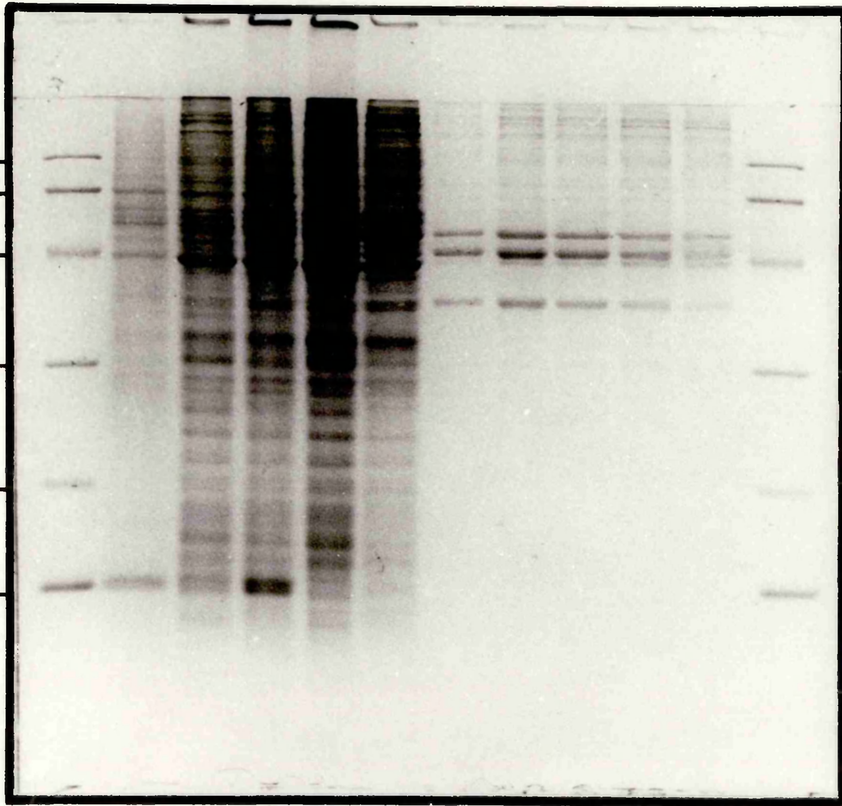
Partially purified BCOAD complex (PEG II clarified material, section 5.2.1) was applied to a column (2.6 x 5.5cm) of Bio-Gel HTP equilibrated with 30mM potassium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT. After washing the column at 24ml/h with 50 bed volumes of 200mM potassium phosphate, pH 7.3, 0.1mM EDTA, 0.1mM EGTA, 1mM benzamidine-HCl, 1mM PMSF, 1mM DTT, BCOAD complex was eluted with 350mM potassium phosphate buffer at a flow rate of 24ml/h. Fractions of 4ml were collected, on which were determined protein content (O—O) as in section 2.2.2 and overall BCOAD complex activity (●--●) as in section 2.2.1.



Enzyme activity (U/fraction)
Protein (mg/fraction)
Fraction number
2.5
2.0
1.0
0
3.5
3.0
2.0
1.0
0
1
5
6
7
8
9
10
11
12

Fig.5.2 **SDS/Polyacrylamide Gel Electrophoresis Of Samples From Stages In The Purification Of Bovine Kidney Branched Chain 2-Oxoacid Dehydrogenase Complex**

Samples from various stages of the purification procedure described in section 5.2.1 were analysed by electrophoresis on a 12.5% (w/v) SDS/polyacrylamide slab gel followed by staining of the gel with Coomassie blue. Lanes 1 and 12, low M_r markers (60µg); lane 2, 1st supernatant fraction (60µg); lane 3, PEG I (60µg); lane 4, PEG I clarified (60µg); lane 5, PEG II (60µg); lane 6, PEG II clarified (60µg); lanes 7-11, consecutive Bio-Gel HTP eluate fractions from peak of BCOAD activity (10µg).



$M_r \times 10^{-3}$
92
67
45
30
21
14
E2
E1α
E1β
1 2 3 4 5 6 7 8 9 10 11 12

M_r values of these bands were estimated to be 52500 ± 1000, 44700 ± 1000 and 37200 ± 1000 (Fig.5.3). In order of decreasing M_r value, the three major components were identified as the BCOAD complex E2, E1 and E1β polypeptides respectively, by comparison with the purified complex described by Pettit et al. (1978) and Lawson et al. (1983).

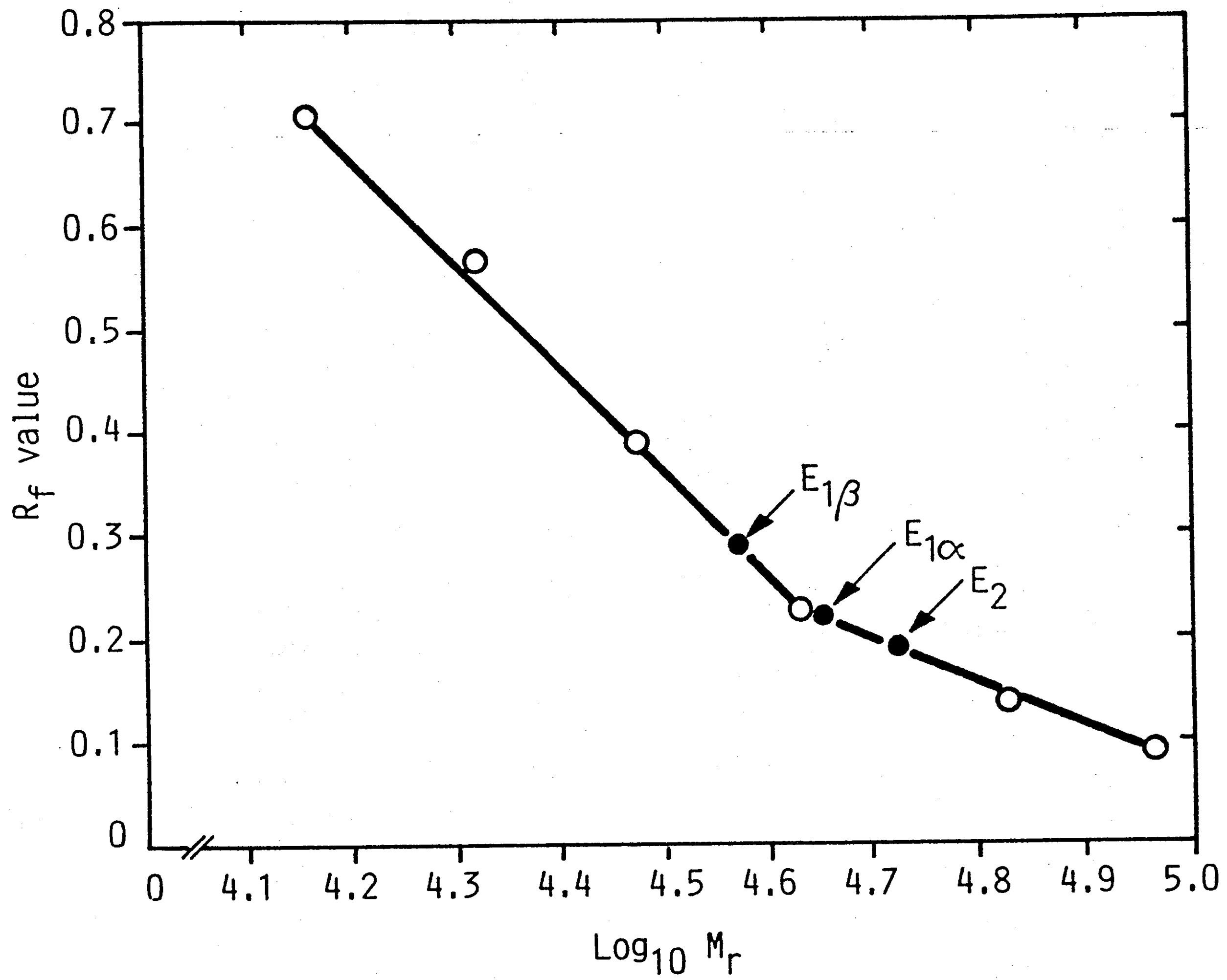
The purity of the individual fractions of BCOAD complex eluted from the hydroxylapatite column (see Fig.5.2) was assessed by densitometric scanning of the Coomassie blue profiles of these samples. The result of such an analysis for the fraction in lane 7 of this figure is presented in Fig.5.4. The homogeneity of this fraction was estimated to be 96%. The ratio of the peak areas corresponding to the α and β polypeptides of E1 was 2.4:1. Densitometric scanning also revealed the absence of the E3 (lipoamide dehydrogenase) component from the purified complex, since no peak was detectable in the 55000-M_r region of the gel. The absence of E3 was also indicated by the fact that the final product showed no overall BCOAD activity until excess pig heart lipoamide dehydrogenase (Sigma) was added to the assay mixture.

The purified complex was tested for its ability to catalyse the oxidative decarboxylation of 2-oxoisovalerate, 2-oxoisocaproate and 2-oxo 3-methylvalerate. The complex was active towards all three branched chain 2-oxoacids, in the ratio 2.2:1.2:1.0.

A summary of one of the purifications performed during this study is presented in Table 5.1. The specific activity of the final material, using 2-oxoisovalerate as substrate, was 36.4 nkat/mg, in broad agreement with the value reported by Lawson et al. (1983). Although the amount of purified material obtained is low in comparison to that reported by Lawson et al. (1983) (0.64mg/100g tissue versus 3.5mg/100g

Fig.5.3 <u>Determination Of The Apparent M_r Values Of BCOAD Complex Components By SDS/Polyacrylamide Gel Electrophoresis</u>

Samples of purified BCOAD complex and low M_r marker proteins were co-electrophoresed on a 12.5% (w/v) SDS/polyacrylamide slab gel, as described in section 2.2.3. After electrophoresis, proteins were visualised by Coomassie blue staining, as illustrated in Fig.5.2. A standard curve of relative migration (R_f) value versus $\log_{10}$ subunit M_r value was constructed, and from this the apparent M_r values of the BCOAD complex components were determined.



0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R_f value
$E_{1\beta}$
$E_{1\alpha}$
E_2
0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
Log_{10} M_r

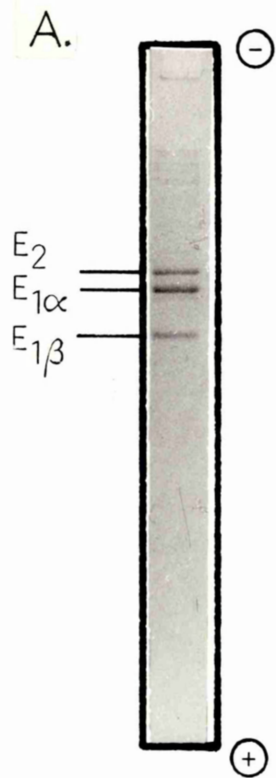
Fig.5.4 Densitometric Scan Analysis Of Purified BCOAD Complex Resolved By SDS/Polyacrylamide Gel Electrophoresis

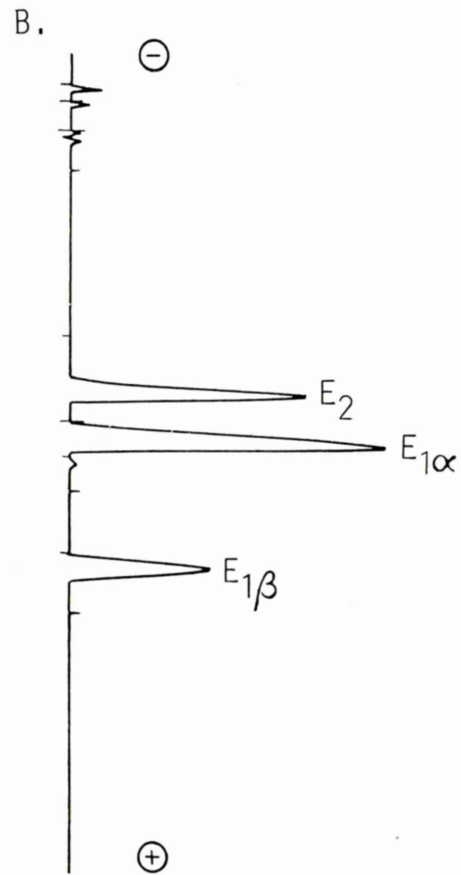
Purified BCOAD complex (10μg) was resolved into its component polypeptides by electrophoresis on a 12.5% (w/v) SDS/polyacrylamide slab gel (section 2.2.3). After staining of protein bands with Coomassie blue, the gel was scanned using an LKB 2202 Ultroscan laser densitometer.

A; Coomassie blue profile.

B; corresponding densitometer trace.

C; table showing percentage total area corresponding to each subunit.





C.

Subunit	% total area
E_2	27
$E_{1\alpha}$	49
$E_{1\beta}$	20
Total	96

Table 5.1 Purification Of Branched Chain 2-Oxoacid Dehydrogenase Complex From Bovine Kidney

FRACTION	VOL (ml)	TOTAL PROTEIN* (mg)	ENZYME ACTIVITY+ (nkat)	SPECIFIC ACTIVITY (nkat/mg)	YIELD (%)
First supernatant fraction	880	13376	---o	---	---
1st PEG precipitate (PEG I)	247	1186	363.1	0.31	100
PEG I clarified	233	699	342.5	0.49	94
2nd PEG precipitate (PEG II)	38	289	86.4	0.30	24
PEG II clarified	31.5	85	75.8	0.89	21
Bio-Gel HTP eluate (total)	24	10.8	114.4	10.60	31.5
40000xg pellets (total)	6	1.4	51.0	36.40	14

All values refer to a preparation from 220g of bovine kidney cortex.
* determined by modified Lowry procedure (see section 2.2.2).
+ using 2-oxoisovalerate as substrate.
o interference from lactate dehydrogenase and NADH dehydrogenase precludes accurate measurement of BCOAD complex activity in the first supernatant fraction.

tissue), the percentages of enzymic activity recovered from the PEG I material are comparable. Thus, the small amount of protein recovered correlates with an approx. six-fold lower level of BCOAD complex activity in the first PEG precipitate. In other preparations, the recovery of purified BCOAD complex per 100g tissue was comparable to or higher than that described by Lawson et al. (1983).

The apparent increase in the total amount of enzymic activity present in the Bio-Gel HTP eluate relative to that in the PEG II clarified material may be due to removal of NADH oxidase activity or an inhibitor of the BCOAD complex during the hydroxylapatite chromatography step.

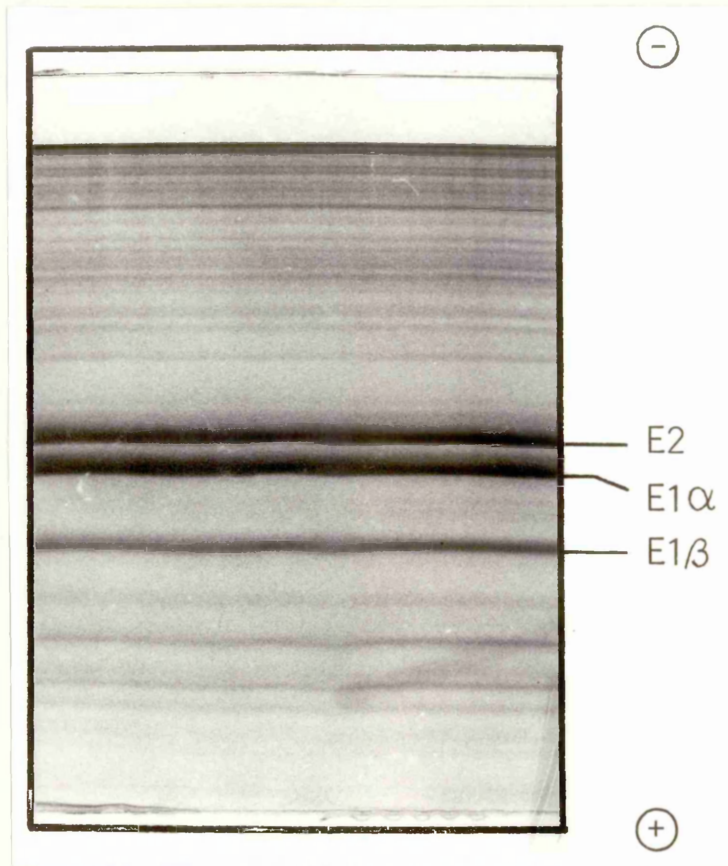
5.3.2 REACTIVITY OF ANTI-BCOAD, ANTI-E1 AND ANTI-E2 SERA WITH THEIR PARENT ANTIGENS

Preparative SDS/polyacrylamide slab gel electrophoresis allowed the resolution of at least 1.7mg of purified bovine kidney BCOAD complex into its E2, E1α and E1β components (Fig.5.5). The Coomassie blue-stained band corresponding to the E2 subunit was excised from the gel and used for the production of subunit-specific anti-E2 serum.

Fig.5.6A shows the Coomassie blue profile of varying amounts of bovine kidney BCOAD complex after electrophoresis on a 12.5% (w/v) SDS/polyacrylamide slab gel, while panels B and C show the results of immune blot analysis when the purified enzyme was probed with anti-BCOAD and anti-E2 sera, respectively. The data in this figure illustrates that both of these antisera show cross-reaction with their parent antigens, although anti-E2 serum exhibits a relatively low titre of antibodies. Examination of the immune blot pattern in panel B

Fig.5.5 Preparative SDS/Polyacrylamide Gel Electrophoresis Of Bovine Kidney BCOAD Complex

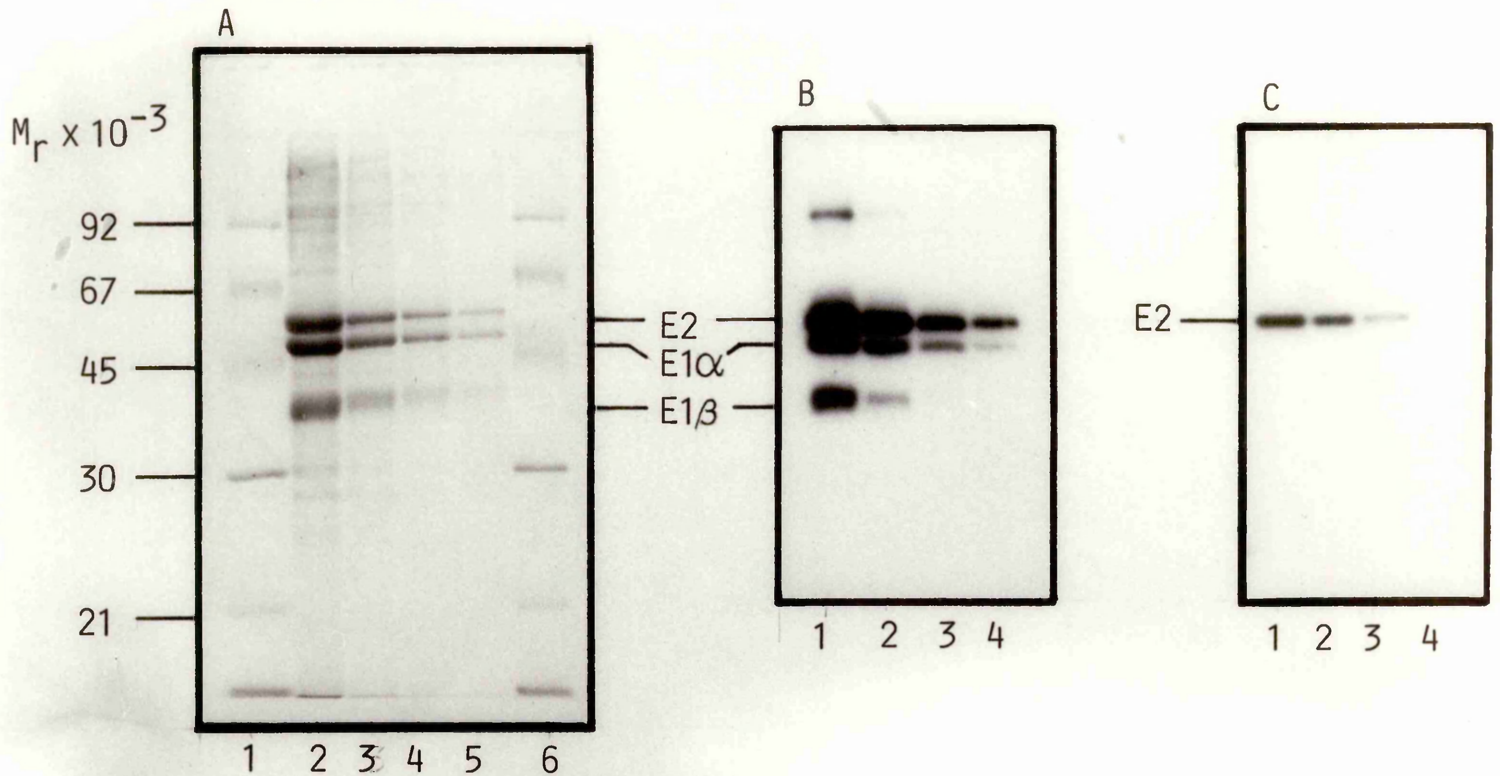
A sample of purified BCOAD complex (approx. 1.7mg) was resolved into its three constituent polypeptides by electrophoresis on a preparative 10% (w/v) SDS/polyacrylamide gel (section 2.2.3). The Coomassie blue-stained E2 band was excised for production of subunit-specific anti-E2 serum as described in section 5.2.2.



−
E2
E1α
E1β
+

Fig.5.6 Reactivity Of Anti-BCOAD And Anti-E2 Sera With Purified Bovine Kidney BCOAD Complex

Varying amounts of purified BCOAD complex were electrophoresed on a 12.5% (w/v) SDS/polyacrylamide slab gel (section 2.2.3). One portion of gel (A) was stained with Coomassie blue. Polypeptides on duplicate portions of gel were transferred electrophoretically onto nitrocellulose paper for incubation with anti-BCOAD serum (B) or anti-E2 (C) serum (section 2.2.8a). Immune complexes were detected by autoradiography (section 2.2.5d) following incubation with ^{125}I-labelled protein A. Panel A; lanes 1 and 6, low M_r markers; lanes 2-5, 25, 10, 5 and 2.5μg of enzyme, respectively. Lanes 1-4 in B and C contain 5, 2, 1 and 0.5μg of enzyme, respectively.


A
B
C
Mr x 10-3
92
67
45
30
21
E2
E1α
E1β
E2
1 2 3 4 5 6
1 2 3 4
1 2 3 4

reveals that anti-BCOAD serum exhibits a weak cross-reaction with the E1β subunit of the complex, relative to the reaction with the E1α and E2 subunits. In addition, at higher loadings of enzyme (Fig.5.6B, lanes 2 and 3), this antiserum also cross-reacts with a high-M_r band, which could feasibly represent an aggregate of BCOAD complex components. However, this high-M_r immunoreactive species did not interfere with subsequent biosynthetic studies performed with this antiserum (see Chapter Six).

Fig.5.7 shows the reactivity of anti-E1 serum with its parent antigen the purified BCOAD E1 subcomplex. Panel B illustrates that this antiserum also showed cross-reaction with both subunits of E1, although the reaction with the E1β polypeptide was again relatively weak. It is worth noting at this stage that anti-E1 serum did not exhibit reactivity with an M_r 60000 band in the preparation of E1 subcomplex used as antigen, although such a band was efficiently immunoprecipitated by this antiserum from detergent extracts of [^{35}S]methionine-labelled PK-15 and NBL-1 cells (see section 6.3.3).

5.3.3 IMMUNOLOGICAL DETECTION OF THE BCOAD COMPLEX COMPONENTS IN SUBCELLULAR FRACTIONS FROM CULTURED CELLS

The antisera raised to the various components of the bovine kidney BCOAD complex were tested for their ability to detect the corresponding parent antigen(s) in post-nuclear supernatant and mitochondrial fractions derived from BRL, PK-15 and NBL-1 cell lines (Fig 5.8). Panel A shows the Coomassie blue profile of samples of these fractions after their electrophoretic resolution on a 10% (w/v) SDS/polyacrylamide slab gel. Panels B and C show the results of immune

Fig.5.7 <u>Reactivity Of Anti-E1 Serum With The Bovine Kidney BCOAD E1 Subcomplex</u>

Varying amounts of purified BCOAD E1 subcomplex were electrophoresed on a 12.5% (w/v) SDS/polyacrylamide slab gel (section 2.2.3). One portion of gel (A) was stained with Coomassie blue. A second portion was processed for detection of immunoreactive polypeptides using anti-E1 serum (section 2.2.8a). Panel A; lanes 1 and 6, low M_r markers; lanes 2-5, 25, 12.5, 5 and 1μg of enzyme, respectively. Lanes 1-4 in B contain 5, 2.5, 1 and 0.2μg of enzyme, respectively.

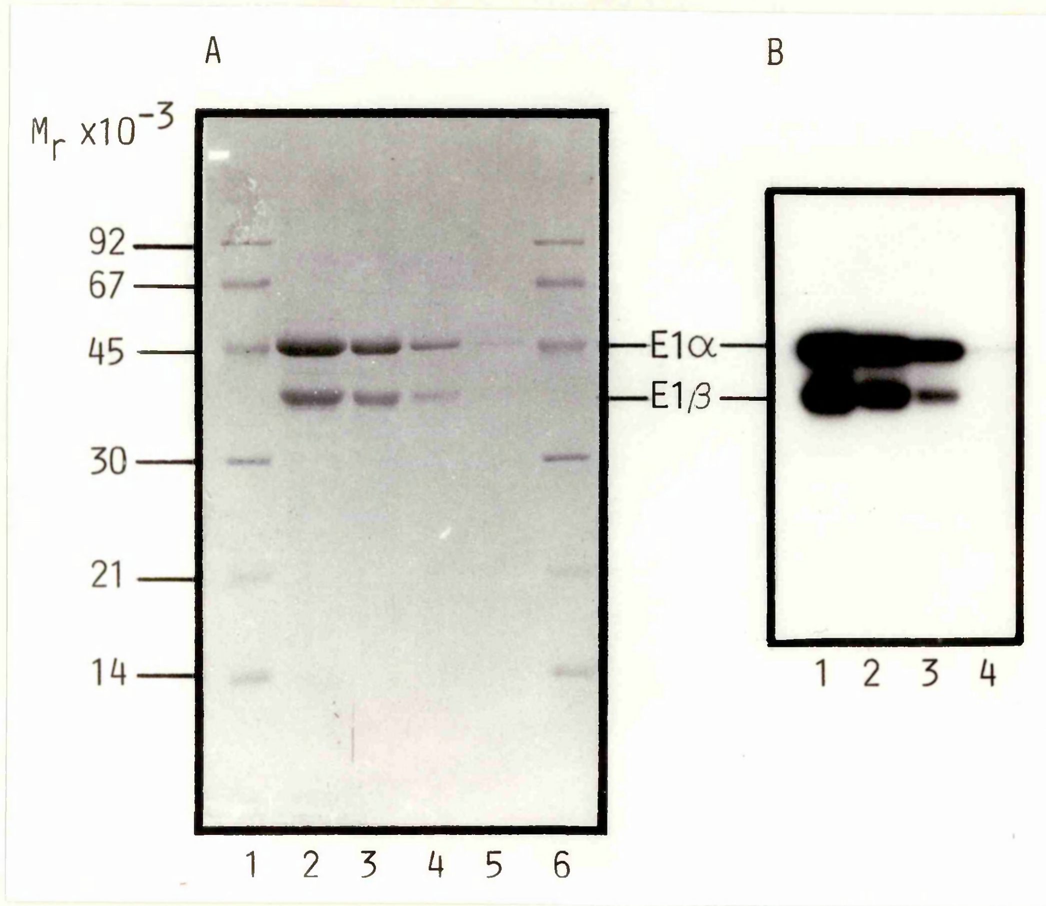
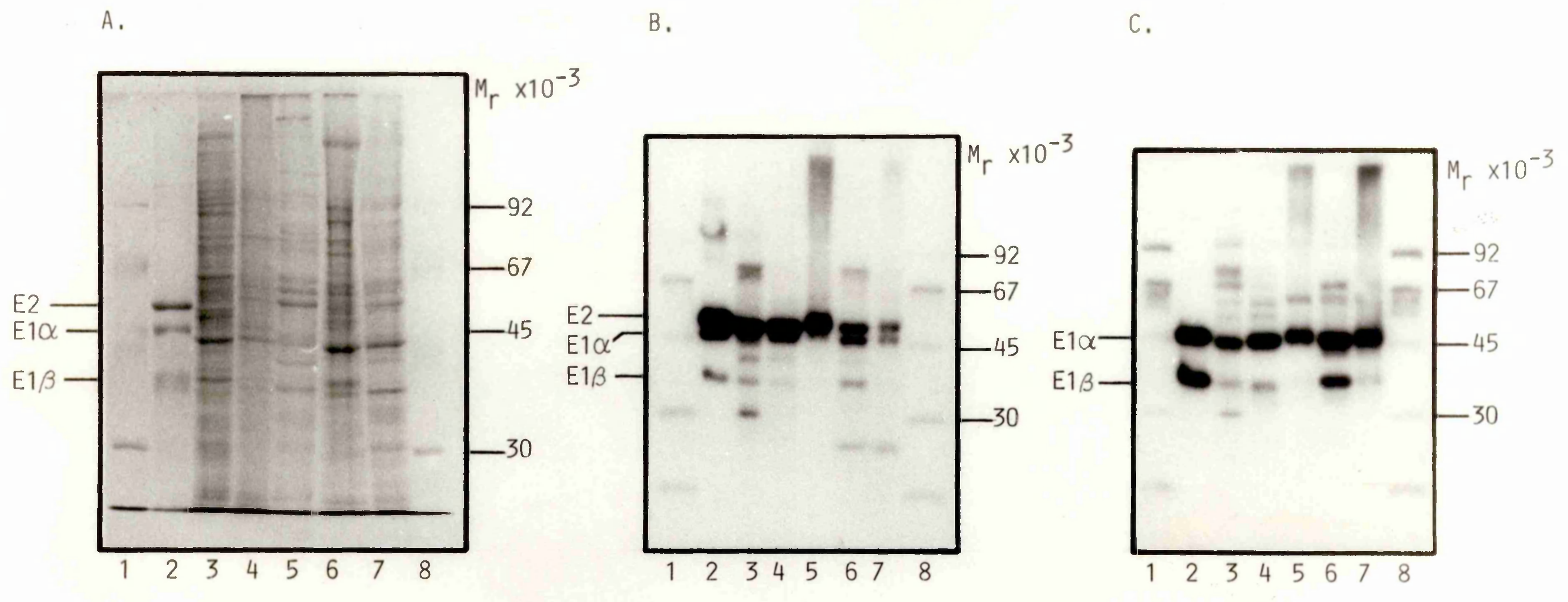

A
B
M_r x10^{-3}
92
67
45
30
21
14
E1α
E1β
1 2 3 4 5 6
1 2 3 4

Fig.5.8 Immunological Detection Of The BCOAD Complex Components In Cultured Cell Subfractions

Post-nuclear supernatant and mitochondrial fractions were prepared from cultured BRL, PK-15 and NBL-1 cells (section 2.2.7), and samples of the extracts were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels. A portion of one gel (A) was stained with Coomassie blue. Replicate portions of gel were processed for detection of immunoreactive polypeptides using anti-BCOAD (B) or anti-E1 (C) serum. Lanes 1 and 8, low M_r markers (A) or ^{125}I-labelled low M_r markers (B and C); lane 2, 10μg (A) or 0.5μg (B-D) purified BCOAD complex; lane 3, BRL post-nuclear supernatant fraction (80μg); lane 4, BRL mitochondria (40μg); lane 5, PK-15 mitochondria (40μg); lane 6, NBL-1 post-nuclear supernatant fraction (80μg); lane 7, NBL-1 mitochondria (40μg).


A.
B.
C.
M_r x10^{-3}
92
67
45
30
E2
E1α
E1β
1 2 3 4 5 6 7 8

blot analysis (section 2.2.8a) when the samples were probed with anti-BCOAD (B) and anti-E1 sera, respectively.

The data in panel B demonstrates that the BCOAD complex E2 and E1 subunits are detectable in each of the three cell lines using anti-BCOAD serum. In contrast, the E1β subunit is detected less easily. Indeed, detection of the band corresponding to E1β in the PK-15 and NBL-1 mitochondrial fractions required overexposure of the blot (not shown). As will become apparent later (see Chapter Six), it is important to stress that anti-BCOAD serum did not exhibit cross-reaction with a 55000-M_r species (i.e. E3, the lipoamide dehydrogenase) in any of the three cell lines.

Fig.5.8C demonstrates that anti-E1 serum also permits detection of BCOAD E1α and E1β in the three cell lines. Again the strength of signal from the E1β subunit was relatively weak. It was also apparent from this blot that anti-E1 serum does not exhibit cross-reaction with the E2 subunit of the purified BCOAD complex (panel C, lane 2).

The E2 subunit of the BCOAD complex in the BRL, PK-15 and NBL-1 cell lines could also be detected when the aforementioned subcellular fractions were probed with anti-E2 serum, although the strength of signal and specificity of reaction obtained with this serum was lower than that obtained with anti-BCOAD serum (not shown).

5.3.4 THE EFFECT OF ANTI-BCOAD, ANTI-E1, ANTI-E2 AND NORMAL (CONTROL) SERUM ON OVERALL BCOAD COMPLEX ACTIVITY

The effect of various sera on the enzymic activity of purified bovine kidney BCOAD complex was investigated. Initial attempts at performing this analysis were hampered by the fact that the purified

complex was found to lose most (approx. 90%) of its activity after incubation for 90 min at room temperature. However, it was found subsequently that enzymic activity was retained after an 18h incubation at 4°C when the purified complex was diluted to a final concentration of 0.2mg/ml with 50mM sodium phosphate, pH 7.0, containing 12mg/ml BSA, 0.2mM TPP, 1mM $MgCl_2$, 2mM DTT and 0.2mM EDTA. Hence the antibody inhibition experiments were performed using the incubation and dilution conditions described above.

Fig.5.9 shows the effects of incubating purified BCOAD complex with increasing amounts of normal (control), anti-BCOAD, anti-E1 or anti-E2 serum. At the highest ratio of serum:enzyme tested (40µl serum:44µg enzyme), anti-BCOAD serum reduced enzymic activity to less than 20% of the initial value. In contrast, enzyme which had been incubated with the same amount of normal serum retained more than 90% of its activity. The effects of anti-E1 and anti-E2 sera were intermediate with respect to those of normal and anti-BCOAD sera, reducing the original enzymic activity by approximately 20% and 30%, respectively.

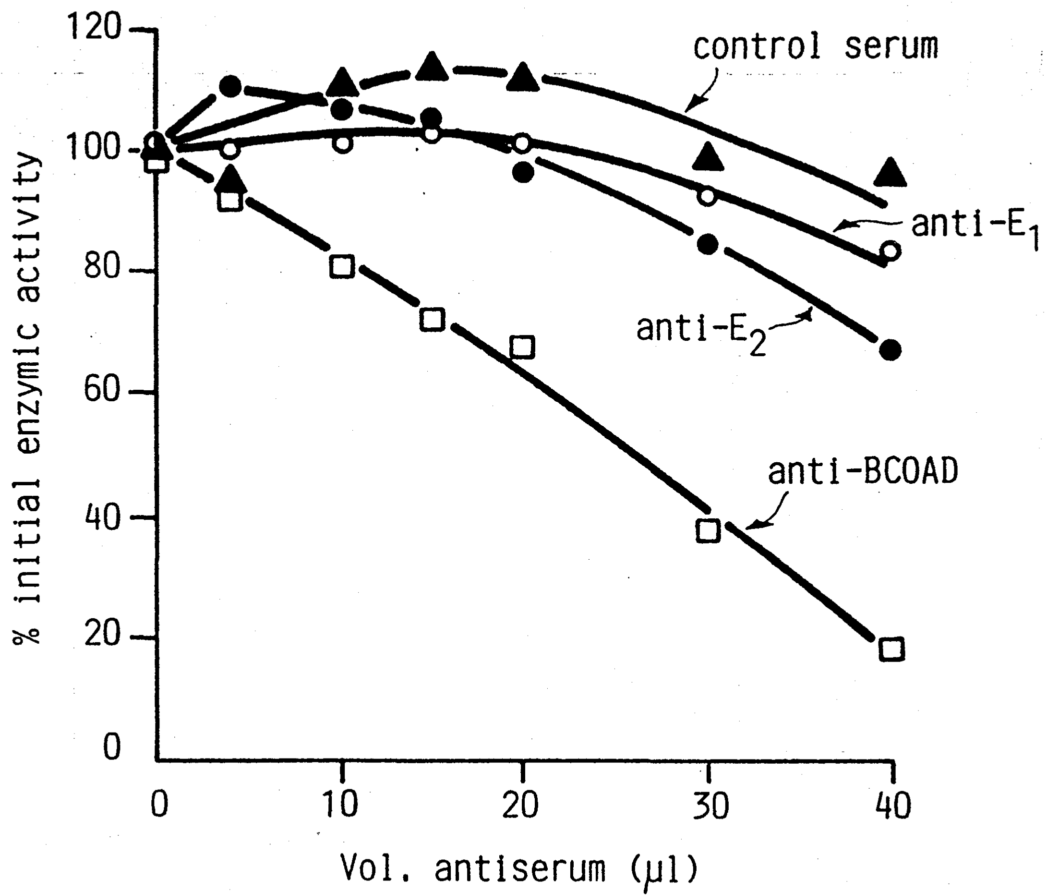
At low ratios of serum:enzyme, anti-E1, anti-E2 and normal sera each caused a small increase in enzymic activity, which was reversed in each case at higher levels of serum. The basis of this apparent stabilisation of the complex is unclear and was not investigated further.

5.4 DISCUSSION

The purified bovine kidney BCOAD complex described in this chapter was similar to that described by Lawson *et al*. (1983) in

Fig.5.9 The Effect Of Various Antisera On BCOAD Complex Activity

Portions of purified bovine kidney BCOAD complex were incubated for 18h at 4°C with varying amounts of normal (▲), anti-BCOAD (□), anti-E1 (o), or anti-E2 (⊗) serum as described in section 5.2.3. At the end of the incubation, duplicate aliquots from each tube were assayed for overall BCOAD complex activity (section 2.2.1). The points represent the means of two determinations, expressed as a percentage of the initial enzymic activity.



120
100
80
60
40
20
0
% initial enzymic activity
0
10
20
30
40
Vol. antiserum (µl)
control serum
anti-E1
anti-E2
anti-BCOAD

terms of polypeptide composition, purity, yield, substrate specificity and specific activity. The complex isolated by Lawson et al. (op. cit.) was reported to contain endogenous kinase activity, capable of phosphorylating the E1α subunit and thus inactivating the complex, although the activity was not assigned to a specific component(s) of the preparation. The presence of such a kinase in the preparation described here was not investigated, since this point was considered to be essentially irrelevant to the present study.

The material obtained included fractions which were sufficiently pure to be used for production of antiserum to the native BCOAD complex as well as fractions which, although of lower purity, could be resolved by preparative SDS/polyacrylamide gel electrophoresis for production of antiserum to the denatured E2 subunit.

The precise reactivities of the antisera produced to the BCOAD complex (anti-BCOAD serum), the native E1 subcomplex (anti-E1 serum) and the denatured E2 subunit (anti-E2 serum) were investigated by immune blot analysis versus their respective parent antigens. Use of this technique revealed that each of these antisera were essentially monospecific. The strength of signal obtained for the E1β polypeptide with both anti-BCOAD and anti-E1 sera was low in comparison with the signal obtained for E1α with these sera. The difference could, in theory, be partially accounted for both by the smaller size of the E1β polypeptide (E1α, M_r = 46000; E1β, M_r = 35000) or by the material used as antigen in this blot containing a molar excess of E1α relative to E1β, although it is likely that the difference in signal strengths arose at least in part through the E1β polypeptide being less immunogenic. A similar situation has been observed with antiserum raised to the native form of the analogous pyruvate dehydrogenase

complex from bovine kidney (Sheu et al., 1985).

The low titre of E2 antibodies in anti-E2 serum relative to anti-BCOAD serum can be rationalised on consideration of the modes of production of these two antisera. Production of anti-E2 serum relied upon injection of small amounts of antigen which was in a denatured and monomeric form. In contrast, anti-BCOAD serum was produced using larger amounts of native complex ($M_r = 3.2 \times 10^6$) which would contain three-dimensional antigenic determinants. Since the immune response is in general enhanced by a physically larger immunogen which possesses three dimensional antigenic determinants, and is dependent on the amount of antigen administered (Maurer and Callaghan, 1980), it can be seen that the production of a high titre antiserum was favoured in the case of anti-BCOAD serum.

Anti-BCOAD, anti-E1 and anti-E2 sera were tested for their ability to inhibit overall BCOAD complex activity. Although each of these antisera had been shown by immune blot analysis to be specific for their respective parent antigens, only anti-BCOAD serum was capable of significantly inhibiting the enzymic activity of the purified complex. This result can be explained by postulating that the antibodies in these two sera recognise antigenic determinants which are sufficiently distant from sites essential for catalytic functioning, so as not to interfere extensively with enzymic activity. The discrepancy between the antibody inhibition and immune blotting analysis data illustrates one aspect of the usefulness of the latter technique.

Based on the observed cross-reactivity of antisera to bovine heart succinate dehydrogenase with equivalent antigens from heterologous sources (i.e. Buffalo rat liver, pig kidney and bovine

kidney cell lines), it was felt that there was good reason to expect that the antisera raised to bovine kidney BCOAD complex would exhibit similar heterologous cross-reactivity. Since it was known that the aforementioned cell lines maintained high levels of aerobic respiration, the level of succinate dehydrogenase expression in these cells could be expected to be reasonably normal. However, since in cultured cells BCOAD complex activity does not constitute a key requirement for normal cellular energy production, an initial point of uncertainty was whether the cell lines would continue to synthesise appreciable amounts of BCOAD complex. Loss of specific enzyme functions from cells grown in culture is not without precedent, for example the rapid shutdown of synthesis of the mitochondrial urea cycle enzyme carbamyl phosphate synthetase from rat hepatocytes after their dispersal from the intact organ (Raymond and Shore, 1981). However, as illustrated in Fig.5.8, the cell lines in question did in fact continue to synthesise BCOAD complex.

Since the antisera were raised to bovine kidney antigens, and because of the superiority of the immunoprecipitates obtained from pig kidney (PK-15) cells over those obtained from Buffalo rat liver (BRL) cells (see Chapter Three), biosynthetic studies on the BCOAD complex were performed using the bovine kidney (NBL-1) and PK-15 cell lines, as described in Chapter Six.

CHAPTER SIX

BIOSYNTHETIC STUDIES ON THE BRANCHED CHAIN 2-OXOACID DEHYDROGENASE COMPLEX

6.1 INTRODUCTION

Biogenesis of the BCOAD complex can conceptually be divided into the following steps; 1) synthesis of the individual subunits as cytosolic precursors; 2) binding of the precursors to the mitochondrial surface and translocation of these species across both mitochondrial membranes; 3) processing of the precursors, which involves insertion of TPP, lipoic acid and FAD into the E1, E2 and E3 components, respectively; 4) assembly of the mature polypeptides within the inner membrane-matrix compartment to generate a functional high-M_r assembly. Thus, it can be seen that the biogenetic pathway pertaining to this mitochondrial component is relatively sophisticated, and therefore of special interest. However, at the outset of this project it was apparent from the literature that none of the events described above had been characterised.

The *in vivo* strategy which had been employed to investigate the biosynthesis of succinate dehydrogenase, i.e. immunoprecipitation of specific antigens from cells pulse-labelled in the absence or presence of uncouplers of oxidative phosphorylation, was also adopted to perform biosynthetic studies on the mammalian BCOAD complex. These studies utilised the various antisera raised against the bovine kidney BCOAD complex, preparation and characterisation of which was dealt with in Chapter Five.

In addition to describing an investigation of the biosynthesis of the BCOAD complex using the metabolic labelling/immunoprecipitation approach, this chapter examines the feasibility of effecting long-term precursor accumulation in cultured mammalian cells during prolonged incubation in the presence of uncouplers.

6.2 METHODS

6.2.1 PREPARATION OF ANTI-E3 SERUM

The antiserum to pig heart E3 used in this chapter was prepared by Miss Anne Phelps in our laboratory. Commercially purified pig heart lipoamide dehydrogenase (Boehringer) was employed to immunise a New Zealand White rabbit using a regime similar to that described for the production of antiserum to native bovine heart succinate dehydrogenase (section 3.2.1). The rabbit received four injections of E3 (1mg each) in Freund's complete adjuvant before the initial bleeding. Subsequent booster injections of E3 (1mg) were carried out in Freund's incomplete adjuvant. The serum obtained was stored in 1ml aliquots at $-20^{o}C$.

6.2.2 PROLONGED GROWTH OF PK-15 CELLS IN THE PRESENCE OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

$175cm^2$ plastic Roux flasks were each seeded with 50ml of NGM containing PK-15 cells at a concentration of 3.6×10^5 cells/ml. When semi-confluent cell monolayers were observed, the growth medium was removed and replaced with 40ml of NGM containing either 2mM DNP, 10μM FCCP or no addition (control incubation). The flasks were then incubated for a further 40h, with the medium being replaced after 24h. At the end of this period, post-nuclear supernatant fractions from each flask were prepared essentially as described in section 2.2.7b, except that the PBS and Triton-TKM solutions were supplemented with 1mM PMSF and 1mM p-aminobenzamidine-HCl and filter-sterilised before use.

6.3 RESULTS

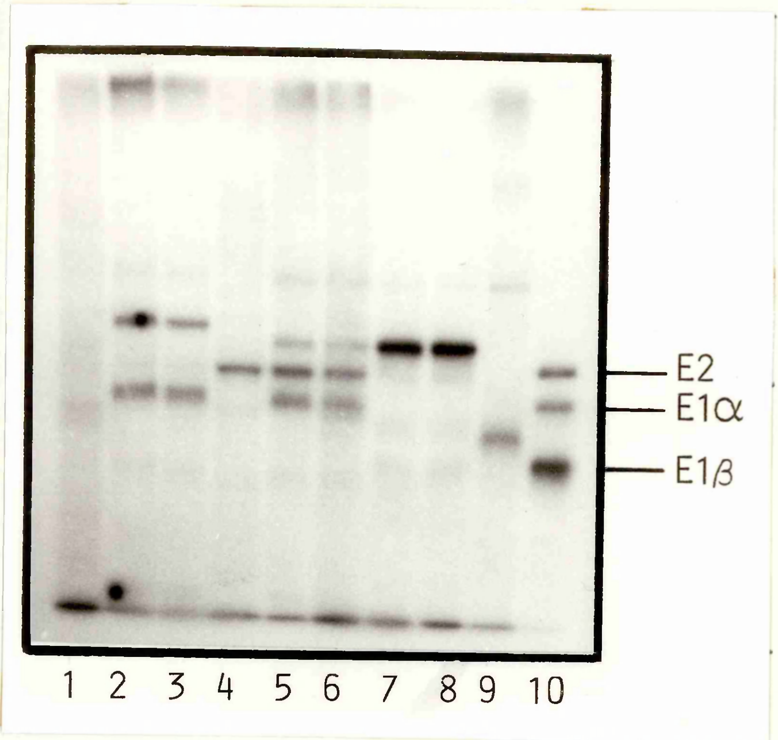
6.3.1 IMMUNOPRECIPITATION OF THE BCOAD COMPLEX COMPONENTS FROM [^{35}S]METHIONINE-LABELLED PK-15 CELLS

The antisera raised to various components of the bovine kidney BCOAD complex were tested for their ability to recover specifically the equivalent antigens from extracts of [^{35}S]methionine-labelled pig kidney cells. Fig.6.1 shows a fluorograph of the products of immunoprecipitation obtained with these antisera, after electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel. Each antiserum was found to effect specific precipitation of the components to which it was directed, as well as certain additional bands, as follows. The immunoprecipitates obtained with anti-E1 serum (lanes 2 and 3) contained, in addition to E1α and E1β, a small amount of E2 subunit, as well as an intense 60000-M_r polypeptide. The identity of this latter band was unclear at this stage, but is discussed further in section 6.3.3. Anti-BCOAD immunoprecipitates (lanes 5 and 6) contained in addition to the E2, E1α and E1β components an extra band which had the same electrophoretic mobility as the E3 component precipitated by antiserum raised to pig heart E3 (lanes 8 and 9).

Although the immune blot analyses described in Chapter Five had revealed that there was no striking differences in the electrophoretic mobilities of the components of purified bovine kidney BCOAD complex and the corresponding components in the pig kidney cell line, differences in electrophoretic mobility were observed between the immunoprecipitated PK-15 BCOAD complex components and the [^{14}C]NEM-modified bovine kidney complex resolved on the same gel. To test whether NEM modification resulted in altered mobility, samples of

Fig.6.1 Immunoprecipitation Of The Subunits Of BCOAD Complex From PK-15 Cells Labelled With [^{35}S]Methionine

PK-15 cells were incubated overnight with [^{35}S]methionine (150μCi/dish) as described in section 2.2.6c. After preparation of [^{35}S]methionine-labelled cell extracts (section 2.2.7c), indirect immunoprecipitation was performed using various antisera in conjunction with formalinised *S. aureus* cells (section 2.2.8b). The resulting immunoprecipitates were resolved on a 10% (w/v) SDS/polyacrylamide slab gel and visualised by fluorography (section 2.2.5c). Immunoprecipitates obtained using; lane 1, non-immune serum; lanes 2 and 3, anti-El serum; lane 4, anti-E2 serum; lanes 5 and 6, anti-BCOAD serum; lanes 7 and 8, anti-E3 serum; lane 9, anti-PDH E1α serum; lane 10, [^{14}C]NEM-modified BCOAD complex. The immunoprecipitate in lane 9 was included as a positive control.


E2
E1α
E1β
1 2 3 4 5 6 7 8 9 10

untreated and NEM-modified bovine kidney BCOAD complex were electrophoresed in parallel on a 10% (w/v) SDS/polyacrylamide slab gel. As illustrated in Fig.6.2, NEM modification did indeed increase the apparent M_r value of each polypeptide. The increase was most pronounced in the case of the E1β polypeptide. Thus, the observed difference in electrophoretic mobilities seen in Fig.6.1 could be accounted for by the effect of NEM modification.

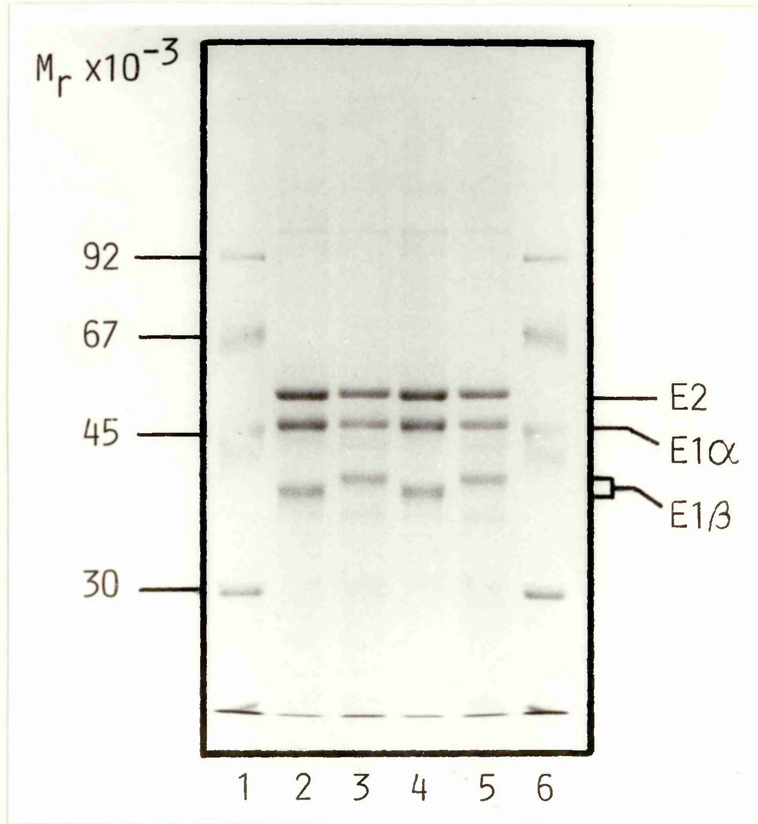
6.3.2 IDENTIFICATION OF THE 55000-M_r POLYPEPTIDE PRESENT IN IMMUNOPRECIPITATES OBTAINED WITH ANTI-BCOAD SERUM

To further investigate the identity of the 55000-M_r band observed in immunoprecipitates obtained with anti-BCOAD serum, the following experiments were performed: 1) an aliquot of [^{35}S]methionine-labelled PK-15 cell extract was heated at 60°C for 5 min before immunoprecipitation with anti-BCOAD serum; 2) 10μg of unlabelled pig heart lipoamide dehydrogenase (E3) was mixed with an aliquot of labelled extract before addition of anti-BCOAD serum; 3) 10μg of unlabelled pig heart E3 was added to an aliquot of labelled extract before addition of anti-E3 serum. The products of immunoprecipitation obtained in each case are illustrated in Fig.6.3.

Lanes 4 and 5 of this figure compare the polypeptide composition of anti-BCOAD immunoprecipitates obtained from control (lane 4) and heat-treated (lane 5) extracts. The profiles obtained in each case were essentially identical, with no apparent loss of the 55000-M_r band after heat treatment. Immunoprecipitation was performed using anti-E3 serum in the absence (lane 7) or presence (lane 8) of competing levels of unlabelled pig heart E3. The addition of unlabelled E3 greatly

Fig.6.2 Effect Of NEM Modification On The Electrophoretic Mobility Of The Subunits Of Bovine Kidney BCOAD Complex

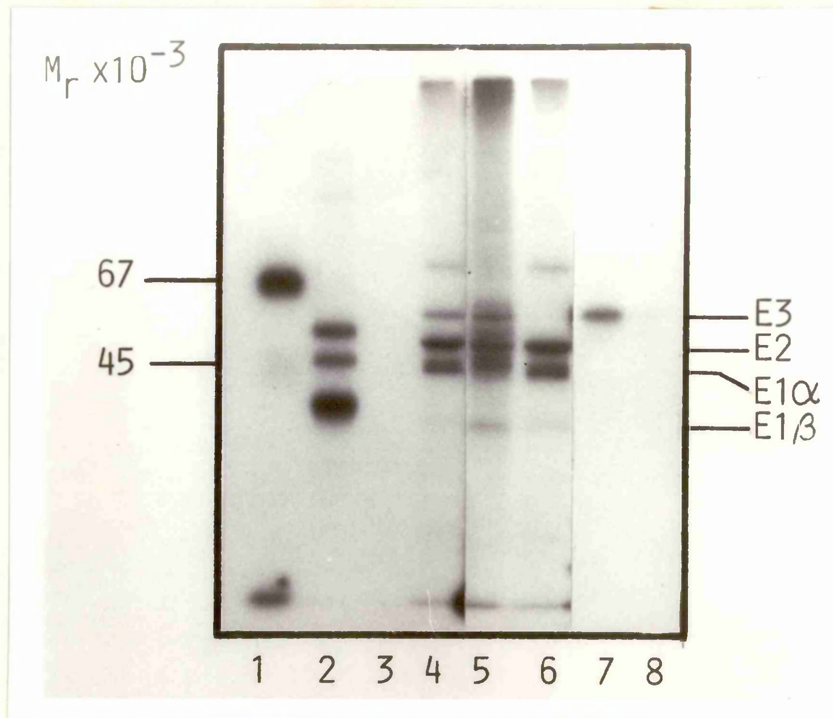
A portion (100μg) of purified bovine kidney BCOAD complex was modified with unlabelled NEM using the protocol described in section 2.2.4a. Samples of this material were electrophoresed in parallel with unmodified enzyme and low M_r markers on a 10% (w/v) SDS/polyacrylamide slab gel (section 2.2.3). After electrophoresis, the gel was stained with Coomassie blue. Lanes 1 and 6, low M_r markers; lanes 2 and 4, unmodified BCOAD complex (10μg); lanes 3 and 5, NEM-modified BCOAD complex (10μg).



M_r x10^{-3}
92
67
45
30
E2
E1α
E1β
1 2 3 4 5 6

Fig.6.3 <u>Identification Of The 55000-M_r Band Present In Immuno-Precipitates Obtained From PK-15 Cells Using Anti-BCOAD Serum</u>

Immunoprecipitation from extracts of PK-15 cells labelled overnight with [^{35}S]methionine (section 2.2.6c) was performed using various antisera. In some cases, aliquots of extract were mixed with 10μg of pig heart E3 or heat treated (60°C for 5min) before addition of antiserum. Products of immunoprecipitation were isolated using formalinised *S. aureus* cells, resolved by electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel, and the radiolabelled polypeptides visualised by fluorography (section 2.2.5c). Lane 1, ^{125}I-labelled low M_r markers; lanes 3-8, immunoprecipitates obtained with the following sera; lane 3, non-immune serum; lane 4, anti-BCOAD serum; lane 5, as 4, using heat-treated extract; lane 6, as 4, plus 10μg E3; lane 7, anti-E3 serum; lane 8, as 7, plus 10μg E3.



M_r x10^{-3}
67
45
E3
E2
E1α
E1β
1
2
3
4
5
6
7
8

diminished the intensity of the E3 band observed on the fluorograph, demonstrating that the pig heart E3 was effective in competing out the equivalent polypeptide from pig kidney cells. When immunoprecipitation with anti-BCOAD serum was preceded by addition of unlabelled pig heart E3 to the extract, the radiolabelled 55000-M_r band was specifically excluded from the immunoprecipitate (lane 6). This result strongly suggested that the 55000-M_r band in question was in fact E3.

The finding that immunoprecipitates obtained using antiserum raised to the native bovine kidney BCOAD complex contained E3 was unexpected, since characterisation of anti-BCOAD serum by immune blot analysis versus its parent antigen or subcellular fractions from cultured BRL, PK-15 or NBL-1 cells had demonstrated that this antiserum did not react with a 55000-M_r species either in the purified complex or subcellular fractions.

Purified bovine kidney BCOAD complex was probed with anti-E3 serum (Fig.6.4) to test for the presence of E3 at the level of sensitivity afforded by the immuneblotting procedure. Anti-E3 serum (1:50 dilution) was capable of readily detecting the E3 component of both the bovine heart OGDC (lane 3) and PDH (lane 6) complexes when 2μg of each were present on the blot. In contrast, when 2μg of bovine kidney BCOAD complex was probed with anti-E3 serum, no band corresponding to E3 was detectable (lane 4). However, when 10μg of BCOAD complex was probed (lane 5), a relatively faint signal was observed.

Anti-BCOAD serum was tested for its reactivity towards pig heart E3 or the E3 component of the bovine heart OGDC and PDH complexes (Fig.6.5). (Samples of purified OGDC and PDH were provided by Dr. Jeff Hodgson from this laboratory). It is apparent from panel B of this

Fig.6.4 Immuneblot Analysis Of Bovine Heart PDH And OGDC Complexes And Bovine Kidney BCOAD Complex Using Anti-E3 Serum

Samples of purified pig heart E3, bovine heart PDH and OGDC complexes and bovine kidney BCOAD complex were electrophoresed on a 10% (w/v) SDS/polyacrylamide slab gel. One portion of the gel (A) was stained with Coomassie blue; the remaining portion was processed for detection of immunoreactive polypeptides using a 1:50 dilution of anti-E3 serum (section 2.2.8a). Panel A: lane 1, low M_r markers; lane 2, pig heart E3 (3µg); lane 3, OGDC complex (10µg); lane 4, BCOAD complex (10µg); lane 5, PDH complex (15µg). Panel B: lane 1, ^{125}I-labelled low M_r markers; lane 2, pig heart E3 (0.5µg); lane 3, OGDC complex (2µg); lane 4, BCOAD complex (2µg); lane 5, µg))AD complex (10µg); lane 6, PDH complex (2µg).

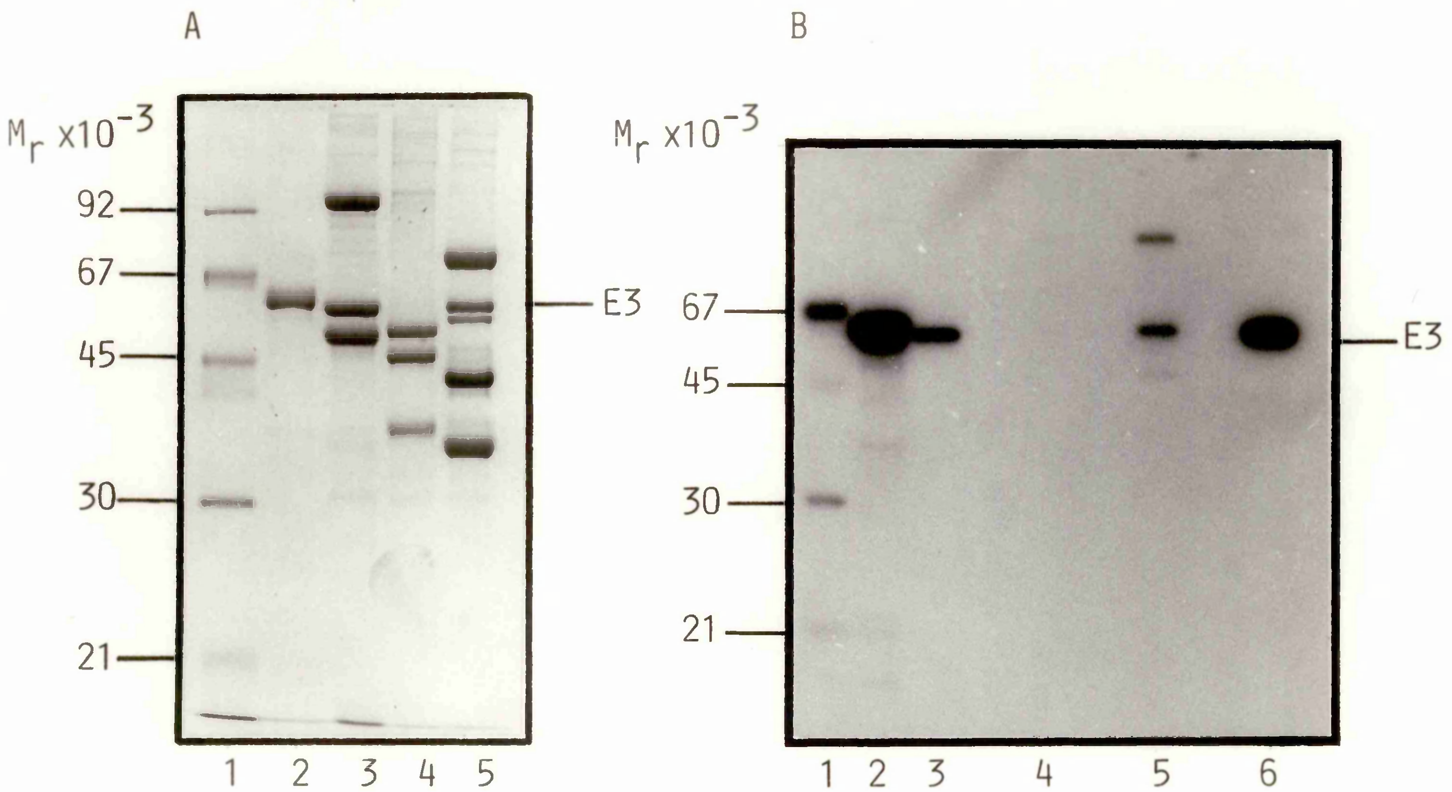

A
B
Mr x10-3
92
67
45
30
21
E3
1 2 3 4 5
Mr x10-3
67
45
30
21
E3
1 2 3 4 5 6

Fig.6.5 Immuneblot Analysis Of PDH, OGDC And BCOAD Complexes And Pig Heart E3 Using Anti-BCOAD Serum

Samples of purified pig heart E3, bovine heart PDH and OGDC complexes and bovine kidney BCOAD complex were electrophoresed on a 10% SDS/polyacrylamide slab gel. One portion (A) of the gel was stained with Coomassie blue. Polypeptides on the remaining portion (B) were electrophoresed onto nitrocellulose paper for incubation with a 1:50 dilution of anti-BCOAD serum (section 2.2.8a). Panel A: lane 1, low M_r markers; lane 2, pig heart E3 (3μg); lane 3, OGDC complex (10μg); lane 4, BCOAD complex (10μg); lane 5, PDH complex (15μg). Panel B: lane 1, ^{125}I-labelled low M_r markers; lane 2, pig heart E3
μg)
(2μg); lane 3, OGDC complex (2μg); lane 4, BCOAD complex (0.5μg); lane 5, PDH complex (2μg). Panel C: blot in panel B after subsequent incubation with anti-E3 serum (1:75 dilution).

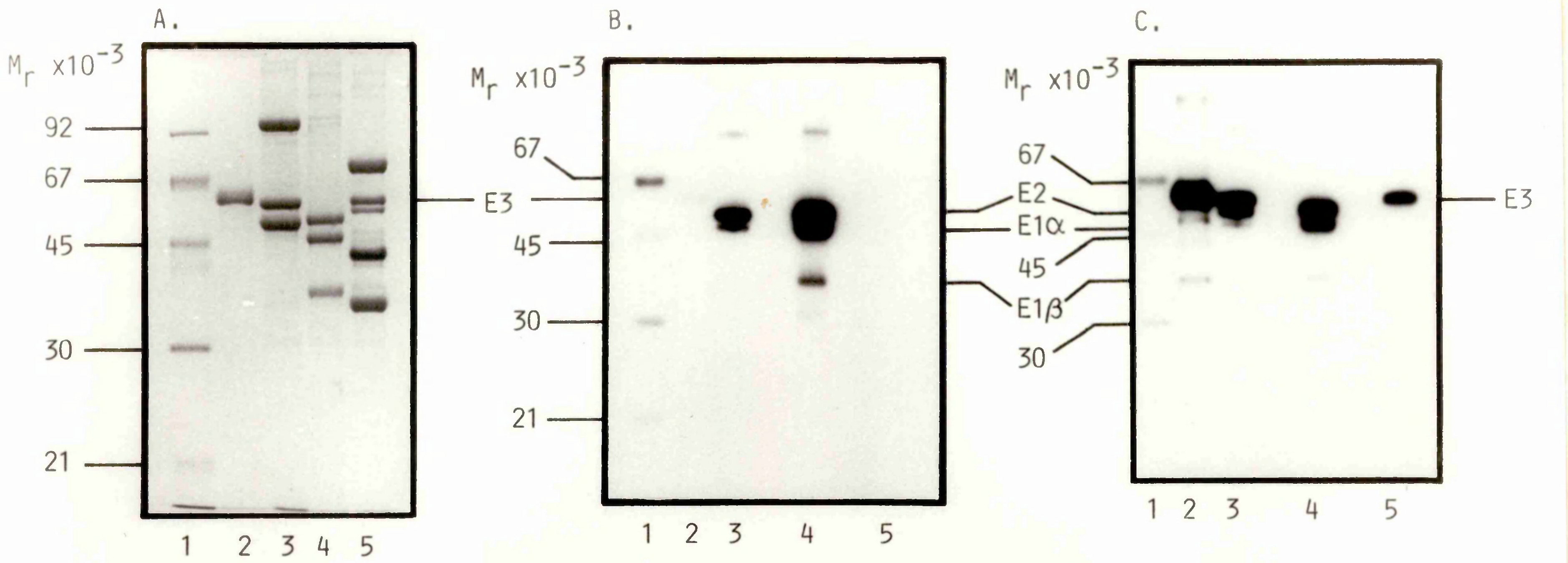

A.
Mr x10-3
92
67
45
30
21
1 2 3 4 5
E3
B.
Mr x10-3
67
45
30
21
1 2 3 4 5
E2
E1α
E1β
C.
Mr x10-3
67
E2
E1α
45
E1β
30
1 2 3 4 5
E3

figure that anti-BCOAD serum did not exhibit reactivity with the bovine heart E3 (lanes 3 and 5) although an extremely faint signal was obtained from 2μg of purified pig heart E3 (lane 2). The blot whose autoradiographic image is shown in panel B was subsequently probed with anti-E3 serum (panel C). The E3 components were readily detected using this antiserum (panel C, lanes 2, 3 and 5).

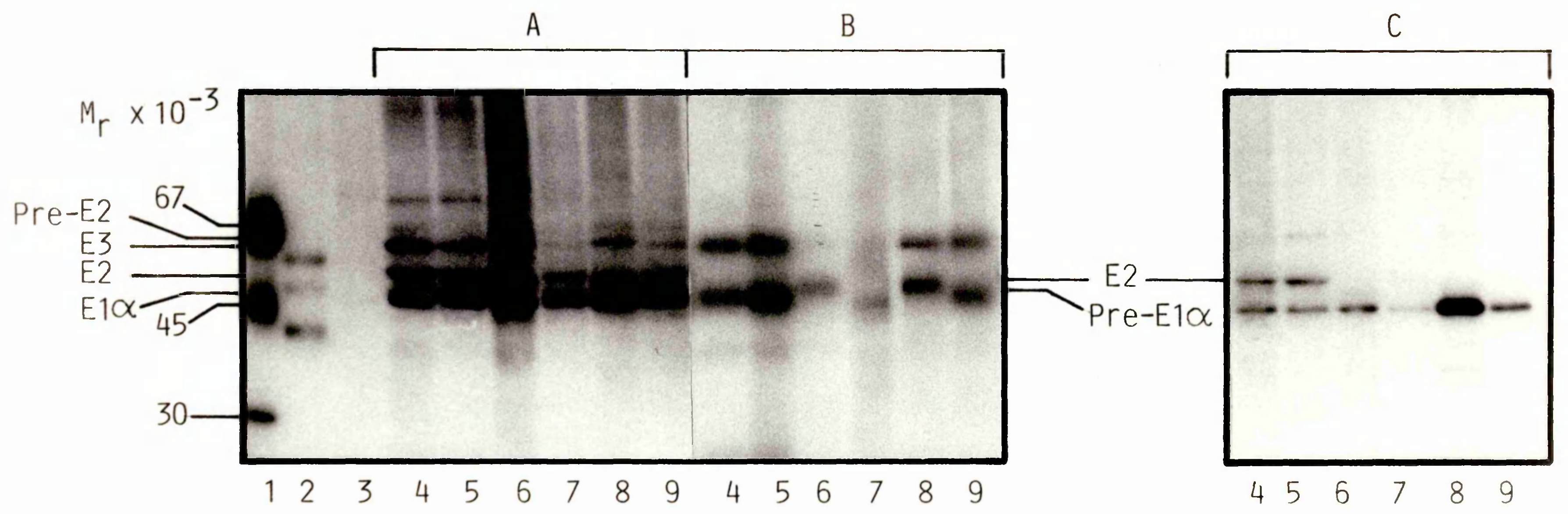
6.3.3 IMMUNOPRECIPITATION OF BCOAD COMPLEX COMPONENTS FROM PK-15 AND NBL-1 CELLS LABELLED WITH [^{35}S]METHIONINE IN THE PRESENCE OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

PK-15 cells were incubated for 4h with [^{35}S]methionine in the presence of DNP (2mM) or FCCP (10μM). In some instances, the cells were then incubated for a further 45 min in normal growth medium minus uncoupler. The polypeptide profiles obtained from extracts of these cells, after immunoprecipitation with anti-BCOAD, anti-E1 and anti-E2 sera, are illustrated in Fig.6.6.

Comparison of lanes 6 and 8 with lane 4 in panel A of this figure reveals that the bands corresponding to the mature subunits of the BCOAD complex are absent in anti-BCOAD immunoprecipitates from extracts of cells labelled in the presence of uncouplers, although a band which exhibits a marginally smaller M_r value than mature E1α (M_r approx. 45000) is observed in these tracks. (The significance of this band is discussed later in this section). In addition, disappearance of mature subunits is accompanied by the appearance of two new bands, with M_r values of 56000 and 49000. These two species are most likely to be the precursor forms of the E2 and E1α polypeptides, respectively. Appearance of a higher M_r precursor form of the E1β

Fig.6.6 Biosynthesis Of BCOAD Complex Components In PK-15 Cells Metabolically Labelled With [^{35}S]Methionine In The Absence Or Presence Of Uncouplers Of Oxidative Phosphorylation

Pig kidney cells were incubated either overnight or for 4h with [^{35}S]methionine (150-200μCi/dish) in the absence of uncoupler, or labelled for 4h in the presence of uncoupler. In some cases, cells which had been pulsed in the presence of uncoupler were subsequently chased for 45 min after removal of uncoupler. After preparation of [^{35}S]methionine-labelled cell extracts (section 2.2.7c), indirect immunoprecipitation was performed using various antisera in conjunction with formalinised *S. aureus* cells (section 2.2.8b). The resulting immunoprecipitates were resolved on a 10% (w/v) SDS/polyacrylamide slab gel and visualised by fluorography (section 2.2.5c). Lane 1, ^{125}I-labelled low M_r markers; lane 2, [^{14}C]NEM-modified BCOAD complex; lane 3, overnight label, non-immune serum. Panels A, B and C illustrate immunoprecipitates obtained with anti-BCOAD, anti-El and anti-E2 sera, respectively, from the following extracts; lane 4, overnight label; lane 5, 4h pulse, no uncoupler; lane 6, pulse + 2mM DNP; lane 7, as 6, but with 45 min chase minus uncoupler; lane 8, pulse + 10μM FCCP; lane 9, as 8, but with 45 min chase minus uncoupler.



A
B
C
$M_r \times 10^{-3}$
67
Pre-E2
E3
E2
E1α
45
30
1 2 3 4 5 6 7 8 9 4 5 6 7 8 9
E2
Pre-E1α
4 5 6 7 8 9

polypeptide was not detected using anti-BCOAD serum.

After a 45 min chase in normal growth medium minus uncoupler, reappearance of components with M_r values of 52000 and 46000, corresponding to the processed (mature) E2 and E1α polypeptides respectively, was observed (lanes 7 and 9), confirming that pre-E2 and pre-E1α had accumulated while precursor uptake was inhibited.

Analysis of the immunoprecipitates obtained with anti-E1 serum (panel B) reveals the following: a) anti-E1 serum precipitates the 49000-M_r putative E1α precursor recognised by anti-BCOAD serum (lanes 6 and 8); b) a higher M_r precursor form of the E1β polypeptide is again undetectable; c) the appearance of mature (processed) E1 subunit again parallels the disappearance of the 49000-M_r species, when a 45 min chase in the absence of uncoupler is performed (lanes 7 and 9); d) the efficiently-precipitated 60000-M_r band observed in mature anti-E1 immunoprecipitates (lanes 4 and 5) remains detectable in immunoprecipitates from cells labelled in the presence of uncouplers (lanes 6 and 8). The last finding would appear to suggest that the 60000-M_r band is a cytoplasmically-synthesised component which is not synthesised as a higher M_r precursor; however, the possibility that in the uncoupler-treated tracks this species represents an aggregate form of BCOAD complex precursors cannot be excluded.

The corresponding immunoprecipitates obtained using anti-E2 serum are shown in Fig.6.6C. As noted previously (Fig.6.1), this antiserum was capable of precipitating the mature form of E2 (lanes 1 and 2). When the cells were pulse-labelled in the presence of 2mM DNP (lane 6) or 10μM FCCP (lane 8), the band corresponding to mature E2 was no

longer detectable, although no higher M_r species was observed.

The presence of a band with a similar M_r value to mature-sized E1 in anti-BCOAD immunoprecipitates obtained from uncoupler-treated cells warrants comment. The separate identity of this species from mature E1 is indicated by two independent lines of evidence. Firstly, a similar band is absent from anti-E1 immunoprecipitates obtained from aliquots of the same uncoupler-treated cell extracts, ruling out the possibility that precursor processing is only partially inhibited. Secondly, closer inspection of the anti-BCOAD immunoprecipitates obtained from pulse-chased PK-15 cells (Fig.6.6A, lanes 7 and 9) reveals the presence of two closely migrating bands in the region of the gel corresponding to mature-sized E1 .

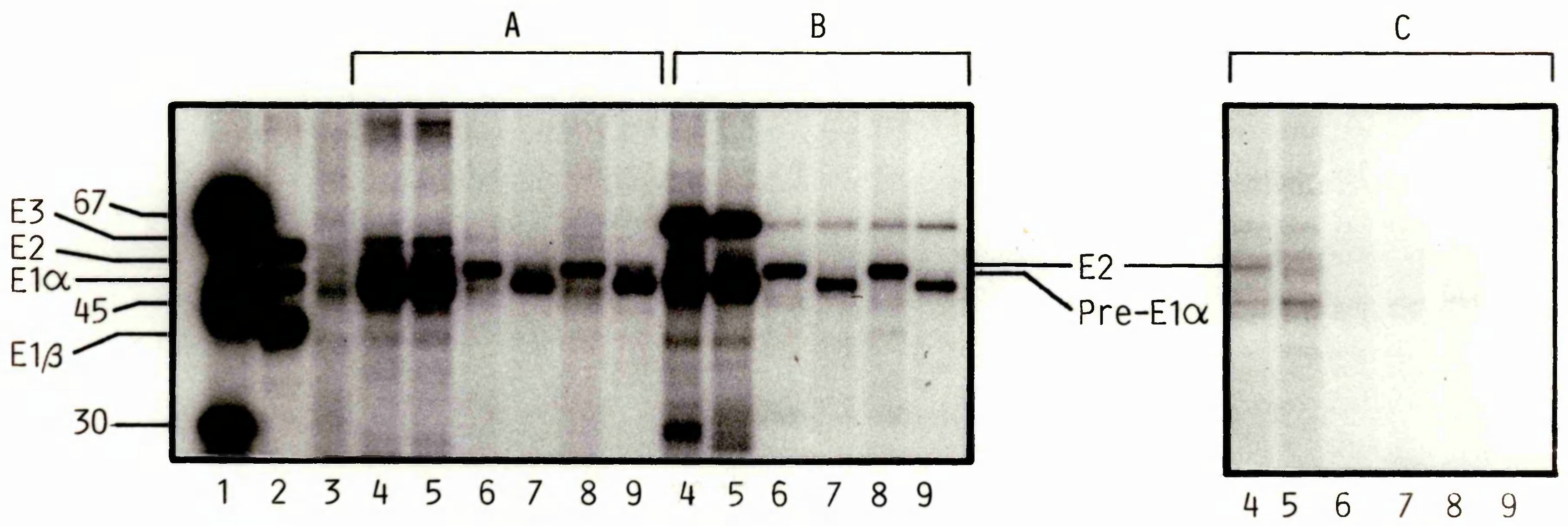
The M_r 45000 band is most probably actin, which has been reported previously to be commonly associated with immunoprecipitates (Barber and Delovitch, 1978).

In a set of experiments analogous to those shown in Fig.6.6, immunoprecipitation using anti-BCOAD, anti-E1 or anti-E2 sera was performed on extracts of bovine kidney (NBL-1) cells which had been pulse-labelled in the absence or presence of uncoupler, or pulse-labelled plus uncoupler then chased for 45 min in medium minus uncoupler (Fig.6.7).

The immunoprecipitates obtained from this cell line resembled their counterparts from the pig kidney cell line in the following respects; a) immunoprecipitates obtained from [^{35}S]methionine-labelled NBL-1 cells using anti-BCOAD serum contained a 55000-M_r species, in addition to the expected E2, E1α and E1β polypeptides; b) the products of immunoprecipitation recovered with anti-BCOAD serum from cells pulse-labelled in the presence of DNP or FCCP contained 56000-M_r and

Fig.6.7 Immunoprecipitation Of BCOAD Complex Components From NBL-1 Cells Labelled With $[^{35}S]$Methionine In The presence Or Absence Of Uncouplers Of Oxidative Phosphorylation

Bovine kidney cells were incubated either overnight or for 4h with $[^{35}S]$methionine (150-200μCi/dish) in the absence of uncoupler, or labelled for 4h in the presence of uncoupler. In some cases, cells which had been pulsed in the presence of uncoupler were subsequently chased for 45 min after removal of uncoupler. After preparation of $[^{35}S]$methionine-labelled cell extracts (section 2.2.7c), indirect immunoprecipitation was performed using various antisera in conjunction with formalinised *S. aureus* cells (section 2.2.8b). The resulting immunoprecipitates were resolved on a 10% (w/v) SDS/polyacrylamide slab gel and visualised by fluorography (section 2.2.5c). Lane 1, ^{125}I-labelled low M_r markers; lane 2, $[^{14}C]$NEM-modified BCOAD complex; lane 3, overnight label, non-immune serum. Panels A, B and C illustrate immunoprecipitates obtained with anti-BCOAD, anti-El and anti-E2 sera, respectively, from the following extracts; lane 4, overnight label; lane 5, 4h pulse no uncoupler; lane 6, pulse + 2mM DNP; lane 7, as 6, but with 45 min chase minus uncoupler; lane 8, pulse + 10μM FCCP; lane 9, as 8, but with 45 min chase minus uncoupler.


A
B
C
E3
E2
E1α
E1β
67
45
30
E2
Pre-E1α
1 2 3 4 5 6 7 8 9 4 5 6 7 8 9
4 5 6 7 8 9

49000-M_r species, which appeared in response to uncoupler treatment; c) the putative E1α precursor (M_r 49000) was also recognised by anti-E1 serum; d) no precursor form of the E1β polypeptide was observed; e) the 45000-M_r putative actin band was observed in anti-BCOAD immunoprecipitates.

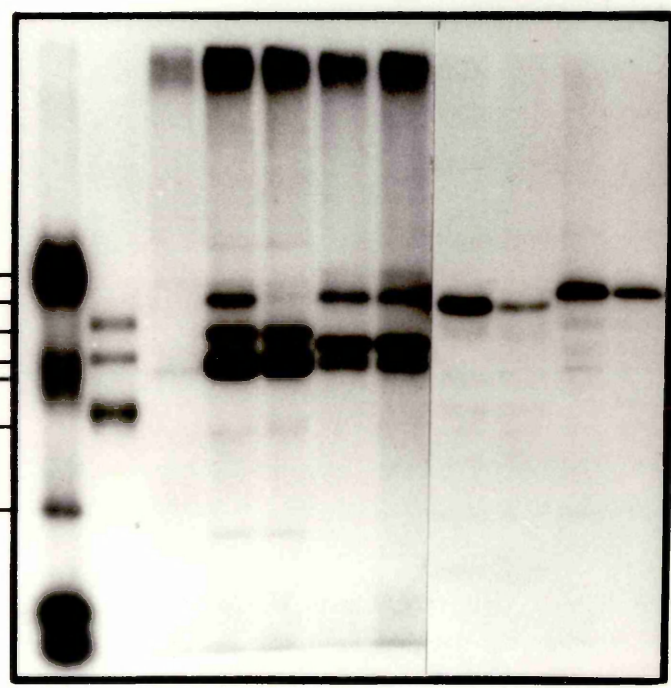
6.3.4 IDENTIFICATION OF THE 56000-M_r BAND OBSERVED IN ANTI-BCOAD IMMUNOPRECIPITATES FROM PK-15 CELLS LABELLED WITH [^{35}S]METHIONINE IN THE PRESENCE OF UNCOUPLERS

Since the lipoamide dehydrogenase of pig kidney cells is synthesised as a precursor of approx. 57000 (De Marcucci *et al.*, 1986b), the possibility existed that the 56000-M_r species observed in anti-BCOAD immunoprecipitates from PK-15 cells pulse-labelled in the presence of uncouplers was in fact pre-E3 (rather than pre-E2), which may have been precipitated because of a hypothetical association with the E2 core component. To investigate this possibility, immunocompetition experiments were performed using purified unlabelled pig heart E3 (Fig.6.8).

As observed previously, (Fig.6.3), addition of purified E3 competed out the 55000-M_r species from anti-BCOAD immunoprecipitates (compare lanes 4 and 5), and the mature E3 component precipitated by anti-E3 serum (lanes 8 and 9). In addition, unlabelled pig heart E3 was observed to compete out pre-E3 precipitated by anti-E3 serum from extracts of PK-15 cells labelled in the presence of 2mM DNP (lanes 10 and 11). In contrast, no effect on the presence of the 56000-M_r species was observed when unlabelled E3 was added to aliquots of extract from DNP-treated, labelled cells, prior to immunoprecipitation

Fig.6.8 Identification Of The Precursor Form Of The BCOAD Complex E2 Subunit In PK-15 Cells

Immunoprecipitates were obtained from [^{35}S]methionine-labelled extracts of PK-15 cells which had been pulse-labelled (4h) in the absence or presence of 2mM DNP using various antisera (section 2.2.8b). In some cases, antiserum addition was preceded by addition of unlabelled pig heart E3 (10μg). Lane 1, ^{125}I-labelled low M_r markers; lane 2, [^{14}C]NEM-modified BCOAD complex; lane 3, 4h pulse (minus DNP), non-immune serum; lanes 4-6, immunoprecipitates obtained with anti-BCOAD serum; lane 4, 4h pulse (minus DNP); lane 5, as 4, plus 10μg E3; lane 6, pulse + 2mM DNP; lane 7, as 6, plus 10μg E3; lanes 8-11, immunoprecipitates obtained with anti-E3 serum; lane 8, 4h pulse (minus DNP); lane 9, as 8, plus 10μg E3; lane 10, pulse + 2mM DNP; lane 11, as 10, plus 10μg E3.



Mr x 10-3
67
E3
E2
E1α
45
E1β
30
Pre-E3
Pre-E2
Pre-E1α
1 2 3 4 5 6 7 8 9 10 11

with anti-BCOAD serum (lanes 6 and 7). This result demonstrates that the 56000-M_r species is immunologically unrelated to E3 and is most probably the precursor form of the BCOAD complex E2 subunit.

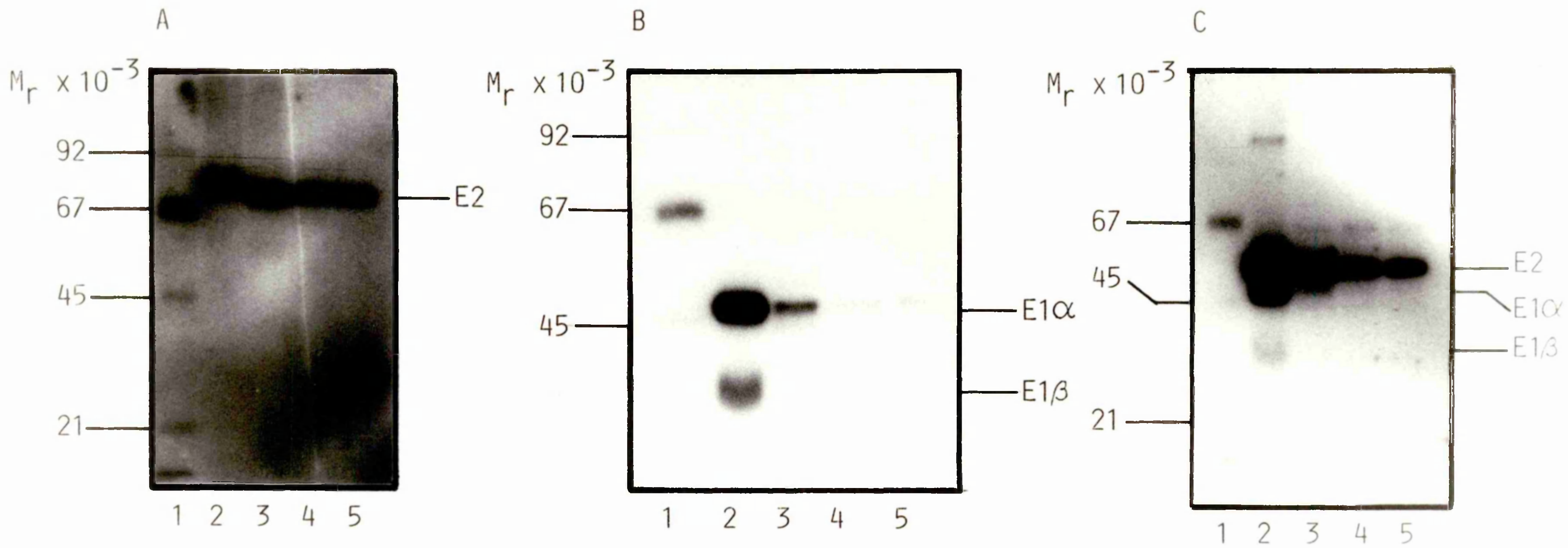
6.3.5 THE EFFECT ON PK-15 CELLS OF PROLONGED INCUBATION WITH UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

As an alternative approach to accumulating precursor forms of the BCOAD complex, PK-15 cells in logarithmic phase were transferred to normal growth medium containing either 2mM DNP or 10μM FCCP and incubated for a further 40h. Cells treated with uncouplers in this way displayed an abnormal morphology when examined under the light microscope and divided at a slower rate than control cells. The uncoupler-induced inhibition of cell growth was reflected by the smaller amounts of total protein recovered from flasks of uncoupler-treated cells relative to a control flask seeded with the same number of cells.

Post-nuclear supernatant fractions (section 2.2.7b) were prepared from uncoupler-treated cells and control cells under conditions which avoided artifactual proteolysis by bacterial or cytosolic proteases or by the matrix processing protease. Samples of these fractions were then analysed by immune blotting (Fig.6.9) using anti-BCOAD serum, anti-E1 serum or subunit-specific antiserum to the bovine heart PDH E2 component (a gift from Dr. O.G.L. De Marcucci), to determine whether precursor accumulation could be detected. The fractions were probed with the latter antiserum since it had been shown previously that accumulated PDH pre-E2 was relatively stable in PK-15 cells maintained in the presence of uncoupler (De Marcucci, 1985).

Fig.6.9 The Effect On Cultured PK-15 Cells Of Prolonged Growth In The Presence Of DNP Or FCCP

Pig kidney cells were grown for 40h in the presence of either 2mM DNP or 10μM FCCP, or in the absence of uncoupler (control incubation) as described in section 6.2.1. Post-nuclear supernatant fractions obtained from cells treated in this manner were prepared and samples of these fractions were subjected to electrophoresis on 10% (w/v) SDS/polyacrylamide slab gels followed by immuneblot analysis (section 2.2.8b) with anti-PDH E2 (A), anti-E1 (B) or anti-BCOAD (C) serum. In each panel, lanes 1, 3, 4 and 5 contain the following samples; lane 1, ^{125}I-labelled low M_r markers; lane 3, post-nuclear supernatant fraction from control cells (40μg); lane 4, post-nuclear supernatant fraction from DNP-treated cells (40μg); lane 5, post-nuclear supernatant fraction from FCCP-treated cells (40μg). Lane 2 contains PDH (0.5μg) in panel A or BCOAD (0.2μg) in panels B and C.



A
$M_r \times 10^{-3}$
92
67
45
21
E2
1 2 3 4 5
B
$M_r \times 10^{-3}$
92
67
45
E1α
E1β
1 2 3 4 5
C
$M_r \times 10^{-3}$
67
45
21
E2
E1α
E1β
1 2 3 4 5

Panel A of Fig.6.9 reveals that when equal amounts of post-nuclear supernatant fractions from control (lane 3), DNP-treated (lane 4) and FCCP-treated (lane 5) cells are probed with anti-PDH E2 serum, a decrease in the intensity of the band corresponding to mature E2 subunit is observed in the extracts from uncoupler-treated cells. However, the appearance of a higher M_r band, corresponding to pre-E2 was not detected in the uncoupler-treated cell extracts. These results suggested that import and processing of newly-synthesised pre-E2 had been inhibited, and that this species had not accumulated in the cytosol, but had undergone degradation in this compartment. Similar effects on the BCOAD complex E2, E1α and E1β polypeptides were observed when the post-nuclear supernatant fractions were probed with anti-E1 serum (B) or anti-BCOAD serum (C).

6.4 DISCUSSION

In recent years, the biosynthesis of mammalian nuclear-coded mitochondrial polypeptides *in vivo* has been investigated by pulse-labelling cultured cells in the presence of uncouplers of oxidative phosphorylation. This experimental approach, which was used in this study to facilitate the detection of higher M_r precursor forms of the large and small subunits of succinate dehydrogenase (Chapter Three), was also employed in an analogous study on the biosynthesis of the mammalian branched chain 2-oxoacid dehydrogenase (BCOAD) complex.

Experience showed that it was relatively difficult to detect the mature-sized subunits of the BCOAD complex, in particular the E1β subunit, in that the immunoprecipitates obtained with antisera to this enzyme contained fewer c.p.m., and thus detection by fluorography

required relatively long exposure times. This difficulty can be accounted for in theory by the relatively low abundance of this multi-enzyme complex in the cell lines studied, and/or by the constituent polypeptides of the E2 and E1 components having amino acid compositions which are low in methionine residues. In the case of the E1β polypeptide, it is likely that the weak anti-E1β reactivity of anti-BCOAD and anti-E1 sera (section 5.3.2) contributed largely to the poor detection of this species.

In an attempt to circumvent any paucity of methionine residues in the polypeptides of interest, cultured PK-15 cells were metabolically labelled with [4,5-^{3}H]leucine. However, any benefit gained from a higher leucine content was offset by the 20-fold lower specific activity of the tritiated radiolabel relative to [^{35}S]methionine, and also because tritium is only poorly detected by fluorography. Although [^{14}C]amino acids (e.g. [^{14}C]leucine) are commercially available, the use of this isotope was not investigated because of cost limitations, and also because [^{14}C]amino acids are poorer than tritiated radiolabels in terms of specific activity.

An unexpected finding of the work presented in this chapter was that immunoprecipitates obtained from extracts of [^{35}S]methionine-labelled PK-15 cells using antiserum to native bovine kidney BCOAD complex contained a 55000-M_r polypeptide which was identified as E3, the lipoamide dehydrogenase, in immunocompetition experiments using unlabelled purified pig heart E3. A polypeptide of similar electro-phoretic mobility was also observed in anti-BCOAD immunoprecipitates from labelled NBL-1 cells, although this polypeptide was not rigorously identified as the bovine kidney lipoamide dehydrogenase.

The above finding was contrary to expectations for two reasons.

Firstly, the material used for production of anti-BCOAD serum was essentially devoid of E3, as determined by absence of lipoamide dehydrogenase activity in the purified preparation, densitometric scanning of the Coomassie blue-stained enzyme and immune blot analysis of the preparation using anti-E3 serum. The lack of E3 in the immunogen made the existence of a substantial titre of antibodies to E3 in anti-BCOAD serum unlikely, especially when the reported low immunogenicity of this polypeptide (De Marcucci *et al*., 1985; Hunter & Lindsay, 1986) is taken into account. Secondly, the predicted absence of E3-specific antibodies in anti-BCOAD serum was confirmed by immune blot analysis when this serum was used to probe the parent antigen (section 5.3.2), subcellular fractions from cultured cells (section 5.3.3), purified pig heart E3 or the E3 components of the bovine heart OGDC or PDH complexes.

The 3D-TKM buffer system (section 2.2.8b) used in these immunoprecipitation studies was selected for its proven ability to promote highly specific precipitation of maximally-dissociated polypeptides from cellular extracts. However, on occasions, incomplete dissociation of polypeptides has been observed even in the presence of this detergent mixture (Hunter, 1985). This finding, taken together with the observed reactivity of anti-BCOAD serum, leads to the hypothesis that the E3 present in anti-BCOAD immunoprecipitates arises through its physical association with the E2 core structure of the BCOAD complex. On this basis, one might expect that heat treatment of the radiolabelled extract prior to immunoprecipitation with anti-BCOAD serum would result in disappearance of E3. Although this was found not to be the case, it is possible to envisage a situation where the association of E3 with E2 subunits is not abolished by heat treatment,

but is sufficiently weak for exchange with added unlabelled pig heart E3 to occur.

The evidence discussed above suggests that *in vivo*, E3 may form an integral part of the BCOAD complex, and that absence of this component from preparations of purified enzyme is probably a result of the isolation procedures used. Isolation of E3-deficient BCOAD complex has been reported for the bovine and rat kidney and bovine and rabbit liver enzymes (Lawson *et al*., 1983; Odessey, 1982; Chuang *et al*., 1984; Paxton and Harris, 1982). A common feature of these procedures is the inclusion of a hydroxylapatite column chromatography step. In contrast, BCOAD complex from bovine liver which has been isolated by a procedure which does not involve hydroxylapatite chromatography contains endogenous E3 (Heffelfinger *et al*., 1983). This finding, coupled with the fact that the lipoamide dehydrogenase from *Escherichia coli* (Coggins *et al*., 1976), *Bacillus stearothermophilus* (Henderson and Perham, 1980) and mammalian sources (Koike and Hamada, 1971) is strongly retained by columns of hydroxylapatite lends support to the argument that the absence of E3 in purified BCOAD complex is a purification artifact.

The biosynthetic studies presented in this chapter provide evidence that the dihydrolipoyl transacylase (E2) and α subunit of the branched chain 2-oxoacid decarboxylase (E1) of the mammalian BCOAD complex are synthesised as higher M_r precursor forms. The third enzyme of the complex, the lipoamide dehydrogenase (E3), which is common to all three mitochondrial 2-oxoacid dehydrogenase complexes, has already been shown to be synthesised as a precursor with an M_r value which is approx. 2000 greater than mature E3.

The putative E2 precursor displayed an M_r value of 56000,

therefore the possibility existed that this species was pre-E3 rather than pre-E2, since the pig kidney E3 precursor is reported to have an M_r value of approx. 57000 (De Marcucci _et al_., 1986b). The recovery of the precursor form of E3 by anti-BCOAD serum (which lacked E3-specific antibodies) could be rationalised by proposing a physical association of pre-E3 with the precursor of the E2 core enzyme, although such an association was considered to be highly unlikely, since it would constitute premature assembly of the complex outside the target organelle. However, the demonstration that the 56000-M_r species in question is not competed out in the presence of purified unlabelled pig heart E3 provides strong evidence that it is the BCOAD E2 precursor, and that this polypeptide is immunologically unrelated to the lipoamide dehydrogenase, but is similar in size to the precursor and mature forms of this component.

The immunoprecipitates obtained from [^{35}S]methionine-labelled extracts of uncoupler-treated pig kidney or bovine kidney cells did not contain a radiolabelled band which could be identified as the precursor form of the BCOAD E1β subunit. The lack of detection of this precursor could be attributed to one or more of the following reasons: 1) synthesis of the E1β subunit is selectively shut down in the presence of uncouplers of oxidative phosphorylation; 2) pre-E1β does not accumulate, i.e. it is rapidly degraded after being synthesised; 3) pre-E1β accumulates during pulse-labelling, but is particularly susceptible to degradation (for example by a serum protease) during the immunoprecipitation procedure; 4) the E1β subunit is relatively poorly labelled by [^{35}S]methionine; 5) pre-E1β is not recognised by antibodies to the corresponding mature polypeptide. In the light of the aforementioned difficulty in detecting mature E1β, it would seem

reasonable to propose that reason 5) listed above is the principal cause of the inability to detect the newly-synthesised E1β subunit.

In 1982, Reid and Schatz reported that when a respiration deficient rho$^-$ strain of *Saccharomyces cerevisiae* is grown for several hours in the presence of the uncoupler CCCP, large amounts of certain mitochondrial precursor polypeptides accumulate in the cytosol of these cells. These precursors could be detected by immune blotting, thus dispensing with the need for immunoprecipitation of radiolabelled antigens (Reid and Schatz, 1982).

With the difficulty experienced in immunoprecipitating the BCOAD complex components in mind, it was felt that it would be of interest to explore the possibility of applying the above method of precursor detection in cultured mammalian cells.

Accordingly, cultured PK-15 cells were subjected to long term incubation in the presence of the uncouplers DNP or FCCP, at concentrations which were known to block import and processing, but allow protein synthesis on cytoplasmic ribosomes to continue at up to 50% of its normal rate. When post-nuclear supernatant fractions prepared from control and uncoupler-treated cells were probed by immune blotting analysis using anti-E1 serum, anti-BCOAD serum or antiserum to the E2 component of bovine heart PDH, no higher M_r species were detected. However, when equal amounts of protein from each sample were probed, a decrease in the amount of mature protein was observed in the uncoupler-treated tracks. These results suggested that import and processing had been inhibited, but without concomitant precursor accumulation, presumably because of instability of these molecules in the cytosol.

In their study, Reid and Schatz (1982) noted that not all

mitochondrial precursor polypeptides could be accumulated during growth of yeast cells in the presence of CCCP. For instance, the cytochrome b_2 and cytochrome c oxidase subunit VI precursors were found not to accumulate under these conditions. Thus, it would appear that in yeast, the precursor forms of different mitochondrial components are differentially predisposed to accumulation in the cytosolic compartment when their import into the mitochondrion is prevented.

Although it is more than four years since the discovery of long term precursor accumulation in yeast, there have been no subsequent reports of the same phenomenon in mammalian cells. Thus, it may be that when mitochondrial import is prevented in mammalian cells, all species of aberrantly-located precursor molecule are subject to proteolytic degradation, so that only a chemically small pool of each precursor exists. Such pools would not be readily detectable by conventional immune blotting procedures.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 BIOSYNTHETIC STUDIES ON MAMMALIAN SUCCINATE DEHYDROGENASE AND BCOAD COMPLEX

As described in Chapter One, the process whereby nuclear coded mitochondrial polypeptides are imported into mitochondria from their cytoplasmic site of synthesis has been the subject of considerable attention in the last eight years.

These recent investigations have elucidated the following general features of the import process. Most imported mitochondrial polypeptides (the main exception being outer membrane components) are synthesised with a transient N-terminal polypeptide extension which has a role in targetting these higher M_r precursors to protease-sensitive binding sites on the mitochondrial surface, and also in subsequent intramitochondrial localisation. Translocation of at least the N-terminal sectors of precursors into or across the inner membrane requires a transmembrane electrochemical potential, and is accompanied or followed by proteolytic processing, i.e. cleavage of the extension sequence in one or two distinct steps. In some cases, processing also involves other posttranslational modifications, such as insertion of cofactors or attachment of prosthetic groups. The ultimate event undergone by a mitochondrial polypeptide during its import is the acquisition of a functional conformation in the correct submitochondrial location.

The present study was concerned largely with the biosynthesis of succinate dehydrogenase and of the branched chain 2-oxoacid dehydrogenase (BCOAD) complex - two areas which have previously received little attention.

Precursor forms of the succinate dehydrogenase large and small

subunits (pre-L and pre-S, respectively) were accumulated and detected in BRL or PK-15 cell lines after pulse-labelling cells in the presence of uncouplers of oxidative phosphorylation. In both cell lines, pre-L and pre-S exhibit M_r values which are 1-2000 and 4-5000 larger than their mature counterparts.

The extramitochondrial precursor form of the large subunit persists for several hours when import is inhibited using DNP, in a form which is capable of being imported and proteolytically processed when the blockade on import is removed. This long term stability contrasts with the differing stabilities of other extramitochondrial precursor polypeptides, which have previously been assessed in a similar manner to that used in this study (Jaussi et al., 1982; Hunter, 1985). Thus, it is highly likely that when import is blocked, pre-L exists in the cytosol in a form which is particularly resistant to degradation by cytosolic proteases.

Weak immunoprecipitation of the small subunit precursor precluded investigation of the kinetics of processing and stability of this polypeptide. The encountered difficulty may be the result of several factors: a) the small subunit is less immunogenic than the large subunit; b) it has a much lower methionine content and c) accumulation of precursor depends on the long term stability of this species in the cytoplasmic compartment.

An analogous biosynthetic study was performed on the mammalian BCOAD complex. Surprisingly, this study revealed that the E3 component coprecipitates with other subunits of the complex using an antiserum which lacks antibodies to E3. This coprecipitation was attributed to a strong physical association between E3 and other component(s) of the complex. This would suggest that in the physiological state, E3 is an

integral component of the BCOAD complex and that its association with the core structure can be disrupted during purification of the complex.

Precursor forms of the E1α and E2 subunits of the BCOAD complex were demonstrated, which exhibited M_r values which are approx. 3000 and 4000 greater than their mature counterparts, respectively. Thus, the precursor of the BCOAD complex E2 subunit is similar in M_r value to the previously characterised precursor form of E3. However, the separate identity of the 56000-M_r putative E2 precursor and pre-E3 was confirmed in immunocompetition experiments using unlabelled purified pig heart E3.

The precursor form of the BCOAD complex E1β subunit evaded detection, most probably because of the difficulty experienced in producing a high-quality antiserum to this polypeptide.

Previous work in this laboratory has identified precursor forms of the component polypeptides of the pig kidney 2-oxoglutarate dehydrogenase (OGDC) and pyruvate dehydrogenase (PDH) multienzyme complexes. As illustrated in Table 7.1, the E2 components of OGDC and PDH are initially synthesised with presequences of M_r 7-9000 and 6-8000, respectively. Thus, these transient extensions are relatively large in relation to the observed size range of mitochondrial presequences (Hay et al., 1984) and are considerably larger than the presequences of the other components of the two complexes. This observation has led to the proposal that the presequence on E2 may contain, in addition to "targetting" and "sorting" signals (Hurt and van Loon, 1986), sequence elements which function to prevent aggregation of E2 subunits before they have been imported into the mitochondrion (Hunter and Lindsay, 1986). The present finding of a 4000-M_r extension on the BCOAD complex E2 precursor would appear to

Table 7.1 Subunit M_r Values For The Precursor And Mature Forms Of The Components Of The Mitochondrial 2-Oxoacid Dehydrogenase Multienzyme Complexes In Pig Kidney Cells

COMPLEX	SUBUNIT	M_r VALUE[a]		M_r EXTENSION SEQUENCE
		MATURE	PRECURSOR	
OGDC[b]	E1	96000	98000	2000
	E2	48000	56000	8000
	E3	55000	56500	1500
PDH[c]	E1 α	42000	44500	2500
	E1 β	36000	39000	3000
	E2	70000	78000	8000
	X	50000	N.D.	N.D.
	E3	as above		
BCOAD[d]	E1 α	46000	49000	3000
	E1 β	37000	N.D.	N.D.
	E2	52000	56000	4000
	E3	as above		

a; determined by SDS/polyacrylamide gel electrophoresis to an accuracy of $\pm$ 1000

b; Hunter and Lindsay (1985).

c; De Marcucci (1985).

d; This study.

N.D.; Not determined.

render this hypothesis less attractive.

7.2 TOPOGRAPHICAL STUDIES ON SUCCINATE DEHYDROGENASE

The topographical arrangement of the large and small subunits of succinate dehydrogenase on the mitochondrial inner membrane was investigated by immune blot analysis of protease-treated bovine heart mitochondria (right side-out; outer membrane removed) or submitochondrial particles (inside-out). In mitochondria, both subunits were resistant to proteolysis provided that the integrity of the inner membrane was preserved, indicating that neither of these polypeptides is present at the cytoplasmic face of the lipid bilayer. This finding is in agreement with those obtained in previous ferricyanide binding, chemical labelling and antibody binding studies (Klingenberg and Buchholz, 1970; Merli *et al*., 1979).

Data obtained in the present study suggests that the large subunit interacts with the matrix side of the inner membrane via two distinct domains, which are detected as separate membrane-associated fragments after treatment of submitochondrial particles with papain or protease K. The lack of detection of a second large subunit fragment after treatment with other proteases (e.g. α-chymotrypsin or trypsin) may arise from the low M_r or lack of immunoreactivity of this species. By the same argument, additional small regions of membrane-associated large subunit may exist, which are also outwith the limits of detection of the technique used.

Girdlestone *et al*. (1981) have investigated the interaction of purified succinate dehydrogenase or complex II with phospholipids by reacting the proteins with lipid vesicles containing radiolabelled

photoactive phospholipids with the reactive moiety in the head-group region (PL II) or on the methyl terminus of one of the fatty acid chains (PL I). The results of these studies led Girdlestone and coworkers to propose that the small subunit of SDH was partially inserted into the bilayer of lipid vesicles, since this polypeptide was labelled by PL II and PL I, whereas the large subunit was shielded from or held above the bilayer since it was not labelled by either probe. Moreover, when succinate dehydrogenase was incorporated into lipid vesicles at below their phase transition temperature and when enzyme-containing membranes were cooled rapidly to below this temperature, the labelling of the small subunit was drastically reduced. This effect is consistent with the protein being excluded from the bilayer in gel-state lipids, but was not seen with complex II, complex III or cytochrome c oxidase, suggesting that with succinate dehydrogenase only a small portion of the molecule enters the bilayer.

The existence of such a model has implications for interpretation of the data of the present study. If the model of Girdlestone et al. (1981) is applicable also to the arrangement of complex II within the mitochondrial inner membrane, the large subunit fragments may be bound to the membrane through interactions with exposed regions of the small subunit (or even the CII-3 and CII-4 components of complex II) rather than through insertion of the large subunit into the hydrophobic interior of the lipid bilayer. Clearly, this point merits further investigation. The inability to detect membrane-associated fragments of the small subunit in the present study does not contradict the model of Girdlestone et al. (1981).

The structural genes for the subunits of mammalian sucinate

dehydrogenase are in the process of being isolated and characterised (T.P. Singer, personal communication). Analysis of the primary sequence of these two polypeptides will facilitate identification of putative membrane-intercalated regions of the two subunits. The topography data presented in this study could then be assessed in the light of such an analysis.

7.3 FUTURE WORK

The biosynthetic studies presented in this thesis provide a foundation for more detailed investigations of the biogenesis of succinate dehydrogenase and the BCOAD complex. It will be of interest to examine the importance of FAD attachment in the import and processing of the SDH large subunit precursor, to determine whether this modification is a prerequisite for proteolytic processing or possibly for assembly of the mature-sized polypeptide into functional enzyme. It is likely that this problem can be tackled in cultured mammalian cells. Since the biosynthesis of FAD from riboflavin involves the incorporation of two phosphate groups from ATP, it should be possible to label FAD by addition of ^{32}P-labelled inorganic phosphate to cultured cells which have been depleted of phosphate and riboflavin. Covalent attachment of radiolabelled FAD could then be monitored by immunoprecipitation using anti-L serum followed by SDS/polyacrylamide gel electrophoresis and fluorography.

The common lipoamide dehydrogenase (E3) component of the three mitochondrial 2-oxoacid dehydrogenase complexes is also a FAD-containing enzyme, although in this case the prosthetic group is bound electrostatically to the polypeptide chain, rather than covalently

attached. Therefore, the application of any approach which monitors FAD incorporation into lipoamide dehydrogenase would have to safeguard against artefactual loss of prosthetic group from the enzyme, for example by denaturation of the polypeptide chain during preparation of samples for SDS/polyacrylamide gel electrophoresis.

The subcellular locations of the enzymes responsible for the attachment of FAD and lipoate to their respective substrates are unknown. However, from the intramitochondrial site of haem attachment to cytochromes c and c_1, it would seem reasonable to predict that the enzymes which perform these modifications are also located within the mitochondrion.

It should be possible to monitor preliminary steps in the assembly of SDH and the BCOAD complex using cultured mammalian cells. This could be accomplished by gel filtration of precursor-containing radiolabelled cell extracts, with subsequent detection of precursor aggregates by immunoprecipitation analysis of the gel-filtered fractions. However, the presence of a precursor polypeptide in a species with a native M_r value which is greater than that of the monomeric precursor is not necessarily indicative of an assembly of precursor molecules. For instance, the precursor polypeptide may be associated with a cytosolic import factor (see section 1.2.4).

The precipitation of the E3 subunit by virtue of its physical association with one or more subunits of the BCOAD complex provides a criterion by which to investigate the kinetics of E3 incorporation. The presence of E3 in anti-BCOAD immunoprecipitates is evidence of physical association of E3 with other BCOAD complex subunits; this can be compared with the appearance of mature-sized E3 which can be monitored using anti-E3 serum. A lag in the appearance of E3 subunit

in anti-BCOAD immunoprecipitates (relative to the appearance of mature-sized E3) would provide evidence for the existence of a pool of processed, but unassembled E3 polypeptides.

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DOCTOR OF PHILOSOPHY

The Degree of Doctor of Philosophy is awarded under Resolution No. 38 of the University Court, which came into effect on 19th November, 1969. The relevant provisions of the Resolution are as follows:

I. The Degree of Doctor of Philosophy (Ph.D.) may be conferred by the University of Glasgow in each of the Faculties thereof.

II. Research students within the meaning of Ordinance No. 350 (General No. 12) who have prosecuted in the University of Glasgow, or in an institution recognised for the purpose by the University Court on the recommendation of the Senatus, a course of special study or research in accordance with the provisions of that Ordinance, may offer themselves for the Degree of Doctor of Philosophy, under the following conditions, namely:

(a) that they have obtained a Degree in any Scottish University, or in another University or College specially recognised for the purpose of this Section by the University Court on the recommendation of the Senatus (provided always that a diploma or certificate recognised in like manner as equivalent to a Degree may be accepted in place of a Degree);

(b) that they have produced to the Senatus evidence of satisfactory progress in the special study or research undertaken by them;

(c) that they have prosecuted such studies on a full-time basis for a period of not less than three academical years, or for such period of part-time study as shall be determined by regulation; provided always that the Senatus shall have power, in exceptional cases, to reduce the period by one academical year, and to permit a research student during part of the period to prosecute elsewhere his special study or research.

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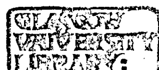
III. All candidates for the Degree of Doctor of Philosophy shall present for the approval of the Senatus a thesis which shall embody the results of the candidate's special study or research, and which shall be accompanied by a declaration signed by the candidate that it has been composed by himself. The Senatus shall appoint a Special Committee which shall always include the Professor or Lecturer in charge of the department concerned; this Committee shall, as the appropriate Faculty shall determine, either examine the thesis or hear the reports of the Examiners, and shall make an appropriate recommendation. The University Court may, on the recommendation of the Senatus, appoint one or more additional Examiners to act along with the Special Committee in adjudicating on the merits of the thesis. The Senatus may, on the recommendation of the Special Committee, require the candidate to present himself for oral or other examination on the subject-matter of his thesis.

IV. The regulations for the Degree shall be as stated in the Schedule hereto.

V. The Degree of Doctor of Philosophy shall in no case be conferred on persons who have not satisfied the conditions hereinbefore set forth, and shall not be conferred *honoris causa*.

VI. Ordinance of the University Court No. 74 (Glasgow No. 21), Regulations for the Degree of Doctor of Philosophy, is hereby repealed.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Regulations

1. A research student may prosecute for the Degree of Doctor of Philosophy a course of special study or research on a part-time basis:

(a) in the Faculties of Law and Financial Studies and Social Sciences, for a period of not less than four years;

(b) in the Faculty of Divinity, for a period of not less than five years;

(c) in the Faculty of Arts, for a period of not less than four years, of which a minimum total period of three months shall be spent in attendance in the University of Glasgow;

(d) in the Faculties of Medicine, Science, Engineering and Veterinary Medicine, for a period of **either** not less than five years of part-time study **or** not less than four years, comprising three years of part-time study and one year of full-time study, provided that the full-time year be spent as one continuous academic session in the University of Glasgow or in an institution recognised for the purpose by the University Court on the recommendation of the Senatus.

A member of the teaching staff of the University, or a person who holds an appointment as Research Assistant or Research Fellow of the University of Glasgow and is paid through the Finance Office or directly by a grant-aiding body approved by the University Court, may notwithstanding offer himself for the Degree of Doctor of Philosophy if he has prosecuted a course of special study or research on a part-time basis for a period of not less than three years.

A member of the teaching or research staff of any other Institution recognised for the purpose of Section II of this Resolution may notwithstanding offer himself as a candidate for the Degree of Doctor of Philosophy in the Faculties of Medicine, Science, Engineering or Veterinary Medicine, if he has prosecuted a course of special study or research in the Faculty for a period of not less than four years part-time.

2. (a) The following Institutions are recognised for the purpose of Section II of the Resolution.

Hannah Dairy Research Institute
Regional Department of Clinical Physics and Bioengineering
Millport Marine Biological Station
Dunstaffnage Marine Research Laboratory
Scottish Research Reactor Centre
West of Scotland Agricultural College
Beatson Institute for Cancer Research
European Nuclear Research Centre (CERN6, Geneva
Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke
Glasgow School of Art
Deutsches Electronen Synchrotron Laboratory (DESY) Hamburg

(b) On the recommendation of the Faculty concerned, and of the Senatus, the University Court may recognise, in the case of individual candidates, other institutions for the purpose of Section II of the Resolution, where satisfactory arrangements can be made for the supervision of the candidate.

(c) A full-time member of staff of a recognised Institution may be nominated to Senate as a supervisor and as an internal examiner or a member of a Special Committee, but not in a sole capacity.

X 8. A candidate for the Degree of Ph.D. must normally expect to be asked to present himself for oral examination on the subject-matter of his thesis.

9. (a) In the Faculty of Arts, if the examiners consider that the thesis has not achieved the standard required for the award of the Degree of Ph.D. but is of sufficient merit, they may allow the candidate to revise and resubmit the thesis for the Degree of M.Litt.

(b) In appropriate cases the Faculty of Social Sciences may recommend that after suitable alteration, some of the material or allied material of a rejected Ph.D. thesis might be submitted for the Degree of M.Litt.

(c) In the Faculty of Divinity, if the Examiners consider that the thesis has not achieved the standard required for the award of the degree of Ph.D. but is of sufficient merit, they may allow the candidate to revise and resubmit the thesis for the degree of M.Th.

10. (a) In the Faculty of Arts, the normal standard of admission will be that of First or Upper Second Class Honours but another qualification, being a qualification approved by the University Court as provided in Section II of the Resolution, may be accepted by this Faculty as being suitable for the programme of study which it is proposed that the applicant should follow.

(b) In the Faculties of Science and Social Sciences, the normal standard of admission will be that of a Degree with First or Second Class Honours of a Scottish University, or an approved equivalent qualification.

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3. In the Faculty of Arts a research student intending to offer himself for the Degree of Ph.D. must obtain the Faculty's approval of his candidature at least one year before applying for the Degree.

4. (a) In the Faculties of Medicine, Science, Engineering and Veterinary Medicine, the period of study or research elsewhere permitted by Section II(c) shall not normally exceed twelve months.

(b) In the Faculty of Social Sciences, although the period of research elsewhere permitted by Section II(c) might exceed twelve months, a candidate must reside in Glasgow for a minimum total period of eighteen months; provided always that the Senatus shall have the powers to vary this requirement on due cause shown.

(c) In the Faculty of Arts, a candidate must be in attendance in the University of Glasgow for a minimum total period of twelve months.

5. (a) In the Faculty of Arts, a candidate must submit his thesis no later than six years from the date of initial registration.[1] The Faculty's Higher Degrees Committee may in special circumstances grant one year extensions on annual application up to a maximum of ten years from admission.

(b) In the Faculty of Divinity, except by special permission of the Senatus Academicus on the recommendation of the Faculty, a thesis may not be presented later than ten years after the date of the candidate's admission to study for the Degree of Ph.D.[2]

(c) In the Faculty of Law and Financial Studies, except by special permission of the Senatus Academicus on the recommendation of the Faculty, a thesis may not be presented later than five years from the date on which the candidate ceases to be a matriculated student.[2]

(d) In the Faculties of Science, Engineering, Medicine and Veterinary Medicine, except by special permission of the Senatus Academicus on the recommendation of the Faculty, a thesis may not be presented later than three years after the date on which the candidate ceases to be a matriculated student.[2]

(e) In the Faculty of Social Sciences the time limit for submission of theses is six years from first registration for full-time students and eight years from first registration for part-time students. Students who have reached the time limit without submitting a thesis may apply annually to the Higher Degrees Committee for an extension of one year, giving reasons for the delay and/or evidence of progress. Any such submissions must be supported by the supervisor or head of department.

[1] In the case of candidates admitted before 1 October 1981, the six-year period shall expire on 1 October 1987, and the ten-year period on 1 October 1991.

[2] This provision shall not apply to candidates admitted prior to session 1982/83.

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6. In submitting a thesis a candidate must state, generally in the preface and specifically in the notes, the sources from which his information is derived, the extent to which he has availed himself of the work of others, and the portions of the thesis which he claims as original. The thesis must be in English.

7. A candidate must submit two copies of the thesis which must include a summary of 250–1000 words. The summary must be an adequate and informative abstract of the work, suitable for publication by the University. The layout and binding of the thesis should generally conform to the British Standard Institution's "Recommendations for the presentation of theses" (BS4821: 1972), a summary of which is available in the University Library or from the Clerk to the appropriate Faculty. Both copies of the thesis, if approved for the Degree, become the property of the University, and are deposited in the University Library.